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305. THE EXCRETION OF BORATE BY THE DAIRY COW

BY E. C. OWEN, *The Hannah Dairy Research Institute, Kirkhill, Ayr*

(With 5 Figures)

In 1942 certain experiments were carried out on the preservative action of borax and boric acid on cattle cakes and similar feeding stuffs. It was felt desirable to determine the fate of such boronated substances after ingestion by the dairy cow. Hove, Elvehjem & Hart (1) had already shown that single doses of boric acid given orally to dairy cows caused a marked increase in the concentration of borate in the milk, but they were only able to recover a small percentage of the dose. Moreover, their experiments shed no light on the boron excretion during prolonged periods of borate feeding.

The experiments described below were designed to ascertain (i) by what route the bulk of the ingested borate was excreted by the dairy cow, (ii) whether prolonged ingestion had any deleterious effect on the milk yield or health of the animal, and (iii) whether the manure from borate-fed animals was likely to contain concentrations of boron which might be toxic to plant life.

OUTLINE OF EXPERIMENTS

Two Ayrshire dairy cows, A and B, were used in the experiments, both being in mid-lactation. The general plan of the experiment was to feed cow A on a control ration and cow B on a ration containing boronated meal. After 42 days the rations were reversed, cow A receiving the boronated ration and cow B the control ration. At the end of 12 days observations on cow B were discontinued, but cow A was kept on the boronated ration for the full period of 42 days, after which she was again fed on the control ration for 13 days. Three comparisons were therefore possible: (i) cow A on the control ration compared with cow B on the boronated ration; (ii) cow A on the control ration compared with her own period on the boronated ration; and (iii) the return to normal of both cows following discontinuance of the boronated ration.

Both animals were housed in metabolism stalls for the duration of the experiment. For the first 8 days the maintenance ration of both cows consisted of hay (14 lb.), cabbages (25 lb.) and swede turnips (25 lb.). Thereafter the ration was altered to 14 lb. hay and 2½ lb. dried beet pulp, a mixture which had a more uniform borate content. The production ration consisted of 1 part each of dried grass, decorticated earthnut meal, bruised oats and bran. This ration was fed at the rate of 4 lb. per gallon of milk. The actual quantities given to each cow were: for cow A, 16 lb. for the first 7 days, and 14 lb. for the remainder of the experiment; for cow B, 18 lb. throughout the experiment. Water was supplied *ad lib.* by automatic water-bowl.

The method of analysis used for boron was a modification of that used by Hove *et al.* (1), the boron being esterified to methyl borate which was removed by distillation with methyl alcohol. The distillate was condensed into caustic soda. The borate so formed was determined colorimetrically by its reaction with quinalizarin in concentrated H_2SO_4,

the colours being compared in a Hilger's Spekker absorptiometer. Details of the method are being published elsewhere. It may, however, be noted that the percentage of borate recoverable by this method is never complete, and varies with the substance examined. Analyses showed that the percentage recovery in the present experiments was 72% with urine and 83% with faeces. With milk, where the boron content is extremely low, the recovery varied from 70 to 120%. However, the borate excreted in the milk never exceeded 3·5% of the total daily output, so that these variations were of no significance in calculating the total balances. A mean recovery of 100% was assumed.

As regards the borate content of the rations, the hay and beet pulp contained 16·5 and 34 p.p.m. of boron respectively, while the control production ration contained 16 p.p.m. The boronated production ration was made by adding 1% of pure borax to the earthnut meal before mixing with the other constituents. It therefore contained 283 p.p.m. of boron. The total daily intake of borate amounted to slightly over 2 g. during the control periods and from 18 to 23 g. during the experimental periods.

Results and discussion

1. *Route of excretion of ingested borate*

The partition of excreted borate in urine, faeces and milk is given in Figs. 1 and 2.

The initial feeding period of cow A shows the uniformly low level of borate* excretion on the control ration containing no added borate. The feeding of the boronated ration was followed, with both cows, by a rapid rise in the borate excretion, which reached a high level in both urine and faeces within a few days. This high level was maintained (with, of course, day-to-day variations) throughout the full period of borate feeding. Cessation of borate feeding after 42 days resulted in a relatively rapid fall to the normal level of excretion, which was attained within about a week.

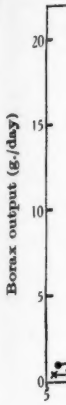
It may be noted that the excretion of borate was somewhat higher for cow B than cow A. This coincides with the higher level of borate feeding of cow B, which was receiving a production ration of 18 lb. in comparison with the 14 lb. fed to cow A.

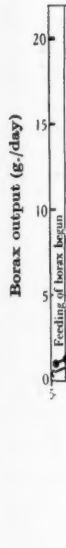
The marked increase in borate excretion in the urine and faeces was paralleled by an increase in the borate content of the milk. This is shown most clearly in Fig. 3, where the scale has been magnified to bring out the differences. Fig. 3 also demonstrates the greater output of borate excreted by cow B, a result due both to the higher concentration of boron and the higher yield of milk.

As regards the distribution of borate between urine, faeces and milk, Figs. 1 and 2 show that the urinary output was practically double the faecal output, a fact which indicates that much of the borax is assimilated into the body, but is then rapidly re-excreted by the kidney. In comparison with the outputs of borate in the urine and faeces, the borate output in the milk was very small. It would seem, indeed, that the appearance of borate in the milk is merely incidental to the presence of increased concentrations of boron in the circulating blood. The increase in the borate content of milk is thus comparable to that which takes place when the blood is flooded with any abnormal substance, e.g. with high concentrations of urea (2).

The above findings are confirmed by reference to the summarized figures for the

* The figures in the present paper are expressed in terms of *borax* except for the analytical results, which are given as p.p.m. of *boron*.





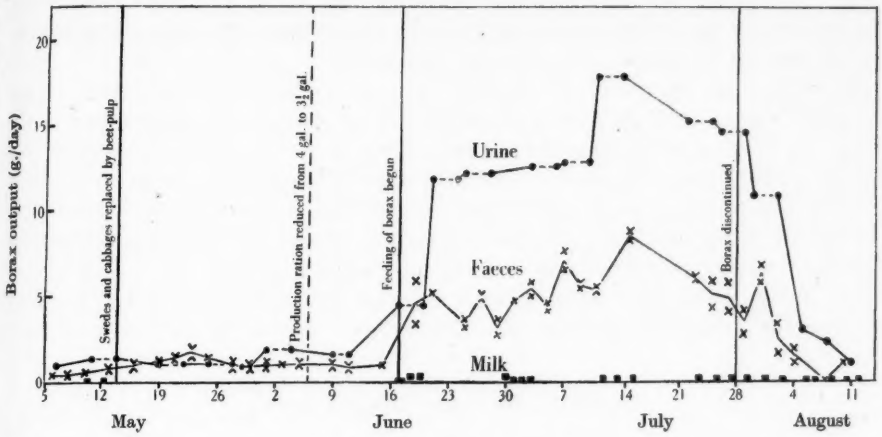


Fig. 1. Borate output of cow A.

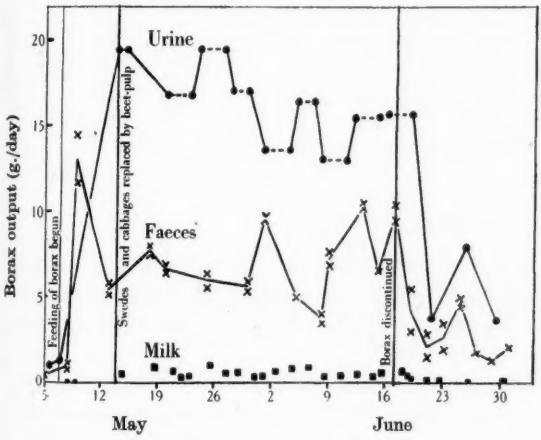


Fig. 2. Borate output of cow B.

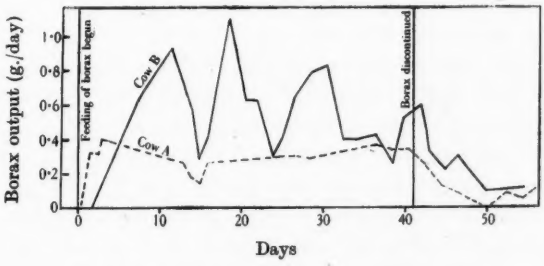


Fig. 3. Borate output in the milk (enlarged scale).

various feeding periods, details of which are shown in Table 1. In arriving at these figures only periods have been used in which there were no changes in the basal ration. The final control period has been subdivided into two parts in order to demonstrate the rate of return of borate excretion on to the normal level.

The outstanding feature of this table is the close balance which exists between the intake and output of borate. The figures in the extreme right-hand column represent the percentage recovery of the ingested borate. It will be seen that they lie between 103 and 110%. Taking into account the considerable errors inherent in the method of analysis, it may be assumed that these figures represent quantitative recovery. There is therefore no evidence of any significant retention of borate in the body. This fact is confirmed by reference to the figures for the final control period, where a return to the normal level of borate excretion took place within about 7 days, i.e. only slightly longer than would be necessary for the complete clearance from the digestive tract of the boronated ration.

Table 1. *Intake and output of borate*

Cow	Period	Days	Output kg./day			Boron content p.p.m.			Output of borate* g./day				Intake of borate g./day	Output as percentage of intake
			Milk	Urine	Faeces	Milk	Urine	Faeces	Milk	Urine	Faeces	Total		
A	Control ration	24	13·75	7·30	28·6	0·7	21·3	4·6	0·09	1·37	1·17	2·63	2·39	110
	Boronated ration	41	11·22	8·73	23·9	2·7	168·8	25·5	0·27	12·98	5·36	18·61	18·13	103
	Control ration (*a*)	7	10·90	8·06	22·7	0·7	107·2	17·3	0·07	7·61	3·45	11·13	2·25	—
	Control ration (*b*)	6	11·40	8·81	22·6	0·4	21·5	2·4	0·05	1·71	0·48	2·24	2·25	—
B	Boronated ration	35	17·98	13·54	32·7	3·7	138·0	24·3	0·59	16·47	7·01	24·07	22·93	105
	Control ration (*a*)	8	16·24	20·75	31·2	1·8	33·0	10·7	0·26	6·03	2·93	9·22	2·52	—
	Control ration (*b*)	4	16·10	20·51	29·5	0·8	20·4	5·7	0·12	3·68	1·49	5·29	2·52	—

* Expressed as borax.

2. *Effect on health and milk yield*

No adverse effects on the health of the animals were observed as a result of the prolonged borate feeding. At the commencement of the experiments the two cows weighed 351 and 361 kg. respectively. At the end of the experiments their weights had increased to 376 and 390 kg.

Records were kept of the yields of urine, faeces and milk during the various feeding periods. Details of these are shown in Figs. 4 and 5. The faecal output remained relatively steady. The urinary output showed marked day-to-day variations, and there were three periods when the water metabolism was exceptional. These periods coincided, however, with abnormal weather conditions, and the results were clearly not related to the boron intake. It may therefore be stated that the borate feeding had no diuretic effect on the cows.

As regards milk yield, Figs. 4 and 5 show that the daily outputs were extraordinarily constant, and that there was no falling off in yield as a result of the borate feeding. It will be noted from Table 1 that the boron content of the milk increased markedly on the boronated ration, i.e. from about 0·7 p.p.m. to about 3·0 p.p.m. This latter concentration is, however, too low to affect adversely the value of milk for human consumption, and would not in fact be measurable by ordinary analytical procedures. The borate content of the milk rapidly fell to the normal level when the feeding of the boronated ration was discontinued.

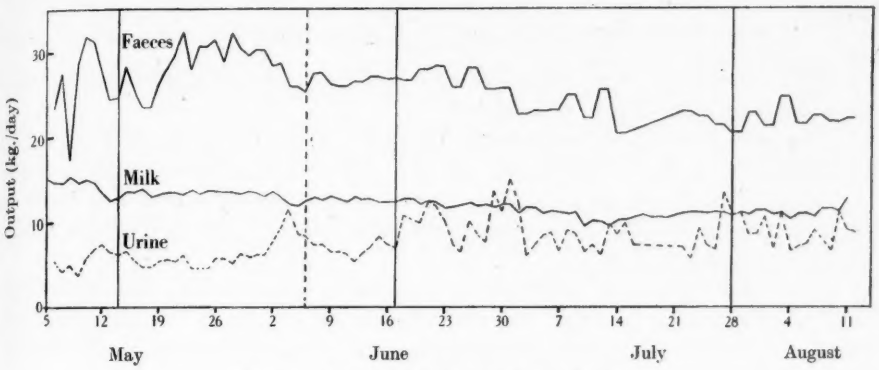


Fig. 4. Yields of milk, urine and faeces of cow A.

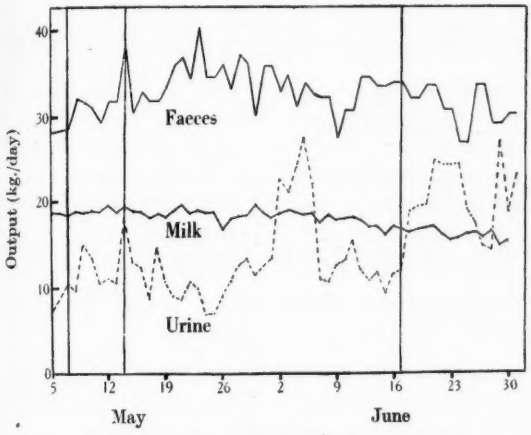


Fig. 5. Yields of milk, urine and faeces of cow B.

Note: The vertical lines in Figs. 4 and 5 have the same significance as those in Figs. 1 and 2.

3. *Concentration of borate in the dung*

Table 1 shows that about 98% of the ingested borate was excreted in the faeces and urine, the borate content of the combined excreta being about 500 p.p.m. Five tons of such excreta would go to the making of some 10 tons of farmyard manure. A dressing of 10 tons per acre of farmyard manure would therefore represent an application of over 5 lb. of borax. While traces of boron are needed for the healthy development of most plants (and would in fact be obtained from normal dung, as shown in Table 1), this element is extremely toxic if present in large amounts. Thus the toxic level for cereals and grassland is generally considered to be 5–10 lb. of borax per acre (4), while dressings of 12–25 lb. are sufficient to cause marked 'yellowing' in beans and other crops (5). Moreover, on certain types of soil the toxic effect of boron is cumulative, and it is practically impossible to eliminate it from such soils once it has been applied. While a single dressing of 10 tons of boronated farmyard manure would probably not be harmful,

repeated dressings at this level would involve considerable risk of permanent damage to the land.

SUMMARY

1. Two Ayrshire cows in mid-lactation were fed for over 40 days on production rations containing from 16 to 20 g. of added borax.

2. The daily excretion of borate during the experimental feeding period was 13–16 g. in the urine, 5–7 g. in the faeces, and 0·3–0·6 g. in the milk, compared with figures of 1·5, 1·0 and 0·1 g. for the control period. There was no detectable retention of borate in the body, and the levels of borate excretion rapidly returned to normal after cessation of the experimental feeding.

3. No adverse effects were observed as a result of the borate feeding. The live weights of the animals remained normal, and there was no diuretic effect. The milk yield was also well maintained. The borate content of the milk increased from the normal level of 0·7 to over 3·0 p.p.m.

4. Attention is drawn to the risk of using farmyard manure which has been made from the excreta of animals fed on boronated rations.

The author wishes to thank Dr N. C. Wright for his advice and interest throughout the investigation.

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(*MS. received for publication* 11 *October* 1943)

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306. A STUDY OF *BACILLUS SUBTILIS* AND RELATED ORGANISMS

By T. GIBSON, *Bacteriology Department, College of Agriculture, Edinburgh*

(With 1 Plate)

An important advance in the taxonomy of the genus *Bacillus* was made in 1936 by the Second International Congress for Microbiology in London. A resolution was approved (1, 2) specifying as the standard culture of the type species, *Bacillus subtilis* Cohn *emend.* Prazmowski (3, 4), a strain which is believed to have been used by Gottheil (5) for his description of the species. The decision to adopt Gottheil's organism as the standard culture could not be expected to meet with universal approval as the recent protest by Lamanna (6) shows. It has, however, finally terminated the confusion, due to the frequent use of the name *B. subtilis* for at least three distinct species, which has made much bacteriological literature practically meaningless. Nevertheless, there is still widespread uncertainty concerning the relationships to *B. subtilis* of the organisms

commonly recognized as *B. mesentericus*, *B. vulgatus*, *B. panis* and *B. globigii*. These bacilli are frequently encountered in several industries: they proliferate rapidly in many foods and, owing to the great resistance of their spores, they come into prominence in canned products and on dairy equipment; certain types may form viscous substances in bread and sugar juices; some produce a rapid disintegration of plant tissues, and some are associated with the fermentation of tobacco. It is desirable, therefore, that their classification should be clarified and their nomenclature stabilized before further confusion arises.

During an investigation of aerobic spore-forming bacilli I have studied 300 strains of *B. subtilis* and closely related organisms. The study was planned with the object of discovering the stable cultural characters and the limits of variation in the species of this group, and the results obtained appear to provide the basis of a satisfactory taxonomy.

SOURCES OF CULTURES

In order to secure a representative series of strains, cultures were isolated from a wide range of sources including soil, dust, manures, silage, hay and other plant materials, milk and other dairy products, dairy equipment, bread and canned foods. To overcome any tendency to select particular types, isolations were made not only by direct plating, but also after preliminary culture in several liquid media at different temperatures and under aerobic and anaerobic conditions. The original materials were often heated, the severity of the treatment varying from a pasteurization at 63° C. to intermittent steaming (40 min. on each of 3 days) and heating in the autoclave for 15 min. at 15 lb. Cultures were also received from the National Collection of Type Cultures and other laboratories. Many of these strains have been propagated on artificial media for long periods, and they are therefore likely to show any changes in character associated with laboratory cultivation.

CRITERIA AVAILABLE FOR DIFFERENTIATING SPECIES

The fact that the genus *Bacillus* contains an enormous number of named species which are difficult to identify is good evidence that unsuitable differential criteria have been used. A brief statement on the significance of certain characters of the organisms is therefore pertinent.

Cultures of *B. subtilis* and related bacilli frequently display exceptionally striking features such as tough pellicles on liquids and wrinkled growths, which may be pigmented, on solid media. The use of such features for characterizing species is attractive but has serious limitations. The properties of the growths appear to depend largely on the amount of gelatinous intercellular material produced, and this attribute varies from strain to strain within a species and among the variants which may be separated from a single strain. It is also influenced by the composition of the medium. For example, cultures of an organism on media containing 1% peptone may appear quite different from those on media containing 0·5%. Potato, which has been extensively used as a diagnostic medium for these organisms, yields especially variable results. Instances of cultural characters which have been found useful are given later in this paper.

Evidence has been obtained that in several species of *Bacillus* biochemical properties are more stable than cultural characters, and they are usually more precise. In our preliminary communications(7, 8) we showed that identification of the organisms is

facilitated if they are grouped primarily according to their action on glucose. Subsequent work has confirmed this finding. Barritt's(9) test for acetylmethylcarbinol after incubation for 7 days has proved most useful. In the *B. subtilis* group (but not in other species of the genus) the elaboration of diastase (tested on plates of nutrient agar containing 0·2% soluble starch, which are flooded with iodine after incubation) and the reduction of nitrate to nitrite have diagnostic significance.

Fermentation tests with sugars and other substances appear to be unsuitable for distinguishing species in the *B. subtilis* group. In media containing peptone the slight initial acidity, which may be produced from several test substances, is soon supplanted by alkalinity owing to a rapid action on the constituents of the peptone. The results are also influenced by the type of medium (agar or broth) and the concentration and kind of nitrogen source. Even if the conditions of the test are standardized strain variation within the species is still found. The apparently clear-cut distinctions obtained by Burdon, Stokes & Kimbrough(10) with these organisms do not apply to every strain studied here.

The manner in which the spore germinates has been suggested—as for example in the recent papers by Lamanna(11, 12)—as an important diagnostic feature of species of *Bacillus*. Nevertheless, when examining a series of strains, including old laboratory cultures, much variation may be found within a single species. It seems likely that the strength and elasticity of the spore wall determine whether the rod emerges from the spore and where and how it does so, or a type of germination occurs in which the spore coat becomes stretched and attenuated so greatly that it can no longer be recognized, a process which is sometimes interpreted as an absorption of the coat. The nature of the spore wall is perhaps associated with other variable properties of spores, such as resistance to heat. Not only are there differences between strains of the same species, but variations may occur depending on the medium used and other conditions of a germination test. These considerations, and the technical difficulty of seeing the details when the spore wall is thin and extensible, appear to preclude the use of spore germination for routine diagnosis.

Characters of the *Bacillus subtilis* group

A sharply defined group of organisms, comprising *B. subtilis* and closely related forms, may be differentiated from other species of *Bacillus* by two features:

(1) *Morphology.* Vegetative cells (Pl. 1, figs. 1, 2) are comparatively small, seldom being over 1 μ in diameter in stained and dehydrated films. When growing on glucose agar they never contain globules of fat (which are unstainable except by specific fat dyes). There is only a slight tendency to form chains. Spores are ovoid to elongate; they produce little or no swelling of the mother cell and are predominantly formed close to its centre.

(2) *Action on glucose.* Acid and acetylmethylcarbinol are produced in glucose broth.

Other characters which may be useful in excluding certain species are: (1) growth occurs readily on the usual bacteriological media in the range 20–45° C.; (2) proteins are actively digested.

This study has indicated that the *B. subtilis* group does not contain more than three types deserving specific rank. They are distinguished in the following paragraphs. Reference will be made only to those characteristics of the organisms which appear to

be the most suitable for differentiating the species and are at the same time easily elicited in any bacteriological laboratory.

BACILLUS SUBTILIS

Of the strains examined 122 have been classified as *B. subtilis* Cohn *emend.* Prazmowski. Some of them differ appreciably in character from the standard culture of this species, but as they are linked by intermediate types it may be inferred that they belong to a single species. Evidence supporting this opinion was obtained by laboratory experiments on variation. From the practical point of view it is perhaps significant that the strains which diverge to the greatest degree from the standard culture were obtained from drastically heated materials. It is not unlikely that difficulties in the identification of such types have contributed to the confusion associated with the bacteriology of canned products.

Differential characters and range of strain variation

Morphology. See characters of the group. In most strains the rods are motile and about 0·7μ thick after staining. In strains which form large amounts of gelatinous material the rods are often about 1μ thick (Pl. 1, fig. 1), motility may be lacking, definite capsules may be formed, and the spores are frequently larger and have a more deeply stainable wall than in other strains.

Colonies. Agar colonies may be round or irregular or spreading. They are easily removed from the medium and are never fimbriate. On ordinary agar the colonies of many strains tend to become thick, yellowish or brownish and wrinkled (Pl. 1, fig. 3). Those of other strains remain thinner and have a mat, dust-like surface and, in transmitted light, a ground-glass appearance (Pl. 1, fig. 4). Less common strains produce smooth glossy colonies (Pl. 1, fig. 5).

Agar slope. On ordinary agar the growth of most strains is at first grey and dull and it tends to spread. The edge is never fimbriate. Later, the growth usually becomes more refractile and yellowish; it may become brownish and rugose, especially if the medium contains more than 0·5% peptone. A wrinkled pellicle forms on condensation water. The growth does not disperse readily in saline or water, and in certain strains it may be viscous or adherent to the agar. In a minority of strains the growth is at all times grey, glossy, smooth and easily dispersed in liquids. Uncommon strains produce a black pigment from certain peptones.

On glucose agar most strains produce a vigorous expansive growth with a ragged edge and a dull surface which often has a dusty appearance. Later, the growth of these strains usually becomes brown and wrinkled and may become more refractile. Less common variants produce a translucent glossy growth. A few strains darken the agar.

Glucose agar stab. A slight stab growth is produced during the first day but becomes less distinct on further incubation (Pl. 1, fig. 8*a*). The surface growth varies in different strains as do their colonies; it is commonly abundant, rugose and brown. Most strains produce a disk of reddish pigmentation in the agar about 1 cm. below the surface. In occasional strains the surface growth and the upper part of the agar become grey-brown or black. (Strains which darken ordinary agar usually produce little or no discoloration of glucose agar, and those which blacken glucose agar do not produce the pigment on ordinary agar.)

Gelatin stab. A stratiform liquefaction to a depth of 1 cm. or more is produced within 7 days at 22° C. (Pl. 1, fig. 9*a*). The liquid may be clear and covered by a tough pellicle, but many strains produce a more fragile pellicle and some turbidity, and a few strains fail to form a definite pellicle. The pellicle may be 'dry' or moist, wrinkled or smooth, and in a few strains it is reddish.

Potato. Many strains produce a dull brown growth raised into thick anastomosing wrinkles (Pl. 1, figs. 12, 13). Others produce a mucoid growth which becomes dull and grey and forms deep, thin, parallel folds (Pl. 1, fig. 14). Less common strains produce a smooth, glossy, grey or yellow growth. The results are greatly influenced by variations in the medium. Certain strains produce a reddish pigment, and in a few the growth and medium become black.

Broth. The type of growth varies from a dull, wrinkled, coherent pellicle resting on a clear liquid, at one extreme, to a uniform turbidity with no pellicle at the other. Most strains form a pellicle.

Milk. Clearing, accompanied by little precipitation of casein, occurs rapidly and the reaction slowly becomes alkaline. Usually little or no curd persists after 7 days at 37° C. (Pl. 1, fig. 10*a*). Uncommon strains produce a faint acidity and they take longer to digest the casein. Reduction of litmus in milk usually occurs at an early stage. Most strains form a pellicle.

Glucose. Acid is formed, but in a peptone medium certain strains begin to produce an alkaline reaction at the surface within 1 or 2 days at 37° C. Acetylmethylcarbinol is produced, but four strains of this series do so in peptone solutions only if these are of unusual composition and only if the tubes are sloped during incubation. They give strongly positive results in O'Meara's (13) medium and on glucose agar slopes. The aberrant strains differ from the others in producing a stronger and more persistent acidity from glucose, but controlling the acidity by neutralization or buffering does not promote the formation of acetoin.

Diastase. Secreted.

Nitrate. Reduced to nitrite by the majority of strains. (Ten of this series fail to produce nitrite.) In a rich medium (e.g. 5% peptone + 5% glucose + 1% KNO_3), made alkaline after sterilization, certain strains produce visible gas, but the gas consists entirely or almost entirely of CO_2.

Oxygen requirement. Aerobic. Not more than a trace of visible growth is produced anaerobically in glucose-containing media.

Temperature requirement. All strains grow at 45° C., the majority at 50° C., and some at 55° C. The minimum temperature for the production from small inocula of a visible growth within 4 weeks is about 12° C.

Pigments. The production of pigments, although an unstable property (see next section), frequently facilitates recognition of this species. The pigments which may be formed are of several types:

(1) The buff or brown colour of growths on solid media is promoted by available carbohydrates and alkalinity.

(2) A brown discoloration of the surface layer of agar, which most strains produce on long incubation, is not influenced by the presence of tyrosine and is suppressed by high concentrations of sugars.

(3) The black pigment produced by certain strains on ordinary agar containing a suitable peptone is due to tyrosinase. Carbohydrates retard and reduce the formation of melanin.

(4) The red pigment which may be formed in glucose agar, glucose broth, gelatin, potato, bread, cereal mashes and other plant products is associated with the presence of carbohydrates and not of tyrosine. Stab cultures in glucose (0·5%) agar show a reddish disk some distance below the surface, but in agar containing 5% glucose, and also in the other media just mentioned, the colour is most intense immediately under the growth.

(5) The black pigment formed by certain strains in glucose agar but never in sugar-free agar tends to appear on the same substrates as does the red pigment. All the strains examined which produce this type of black pigment also form the red pigment. The black, unlike the red colour, is always most intense at the immediate surface of a culture, and different conditions are required for the production of the two pigments.

Variation in laboratory cultures

The foregoing description includes a considerable range of cultural types, but a suitable basis upon which to divide the series of strains has not emerged. It seemed desirable, however, to test the stability under laboratory conditions of some of the salient forms. With this object in view attention was given particularly to strains possessing cultural characters of possible differential significance. Of the methods used for the purpose of inducing variation, cultivation in glucose broth combined with colony selection was the most successful.

Variation in the form of growth and morphology. Strains which do not differ significantly from the standard culture of *B. subtilis* produce on potato a brownish, frequently wrinkled growth, and thus conform to the old conception of *B. mesentericus fuscus* Flügge (14), later designated *B. mesentericus* Trevisan (15) and often referred to as the 'brown potato bacillus', or to the *B. vulgatus* of several writers (16, 17, 18), *B. levaniformans* β Greig

Smith(19) and other named species. Some strains of this type proved to be distinctly stable whereas others readily yielded variants. Most of the variation appeared to occur in two directions leading (*a*) to a loss and (*b*) to a gain in ability to form extracellular gelatinous material.

(*a*) Non-gelatinous variants, in their extreme form, produce smooth glossy colonies (Pl. 1, fig. 5); their growths on solid media are friable and do not become brown or wrinkled and do not acquire a dry appearance; a dense turbidity without a pellicle is produced in broth.

(*b*) In variants which form especially large amounts of viscous material the growth on solid media is very variable in character, often showing both mucoid and 'dry' parts. It may, in the case of the same organism, be either sticky and adherent or easily removable in the form of large coherent flakes. It tends to become markedly rugose or, if sufficient moisture is present, to flow to the base of a tube. On potato wrinkling usually takes the form of deep parallel folds (Pl. 1, fig. 14). In broth a tough pellicle is formed. The bacilli may occur in definite capsules, but more commonly they are embedded in an amorphous mucilage. Organisms of this type possess the characters of the 'potato bacillus', *B. mesentericus vulgatus* Flügge(14), which later received the binomial *B. vulgatus* Trevisan(15), and of *B. panis* (Vogel) Migula(20), *B. subtilis* var. *viscosus* of Chester(21) and Ford, Lawrence, Laubach & Rice(16), *B. viscogenes* Hood & White(22), and possibly of *B. liodermos* Flügge(14). Ford's organism (2587 in N.C.T.C.) is still typical of this type but no longer markedly ropy.

Several of the viscous variants appeared to be non-motile when they were first separated. They seem to possess the characteristics of several previously described organisms of this group such as *Bacterium mesentericum* Migula(20) (*Bacillus mesentericus panis viscosi* I Vogel), *Bacterium panis* Fuhrmann(23, 24), *Bacillus viscosus berolinensis* Seligmann(25), and the *Bacillus panis* described by Ford *et al.*(16) and Spitzer & Epple(26). Absence of motility in these organisms is apparently an unstable condition of no taxonomic importance. Two of the non-motile variants obtained in this work were preserved and were definitely motile when re-examined some months later. Further, von Czadek & Kornauth(27) found Vogel's organism to be motile, while Ford's strain of *B. panis* (2594 in N.C.T.C.) is now actively motile.

The smooth friable form of growth frequently becomes firmly established, but variation has been observed in directions opposite to those already described; and cultures of an intermediate character may persist in a condition of instability, continuing to produce two distinctive types of colony although one is repeatedly selected. The variants obtained from pure cultures duplicate the considerable range of growth forms noted in the original strains and provide evidence of the essential unity of the latter. Variants of pure cultures are frequently asporogenous but so also are occasional freshly isolated strains. In contrast to the impressive changes observed in cultural character, no instance of an alteration of response to the commonly used biochemical tests was detected in this work.

Possibly all the variation described above has been seen before. The papers by Seligmann(25), Gee(17), Kerckhoff(29), Wahlin(18) and Flynn & Rettger(30) give many details, and Morrison & Rettger(31) describe a type of variability which is not uncommon in *B. subtilis*. Owen(32) has shown that the formation of levan from sucrose is also an unstable property of this species. The variants of '*B. mesentericus vulgatus mucosus*'

described by Ivanovics(33) are unlike those of *B. subtilis*; the organism is probably *B. licheniformis*.

Variation in pigment production. Some of the variants separated from strains which form a red pigment produced less colour in suitable media than their parent strain, and in a few cases the ability to elaborate pigment appeared to have been entirely lost. Console & Rahn(34) record a change in the opposite direction. The production of a red pigment on potato has been regarded as the principal diagnostic feature of *B. mesentericus ruber* Flügge(14) or *B. globigii* Migula(20), but as the property is neither entirely stable nor exclusively possessed by strains of *B. subtilis* (see *B. licheniformis*) it has little taxonomic significance.

Constancy in the production of black pigments was also tested, using (*a*) strains showing tyrosinase activity, and previously named *B. lactis niger* Gorini(35) and *B. niger* Migula(20), and (*b*) strains which blacken glucose agar but not ordinary agar, commonly known as *B. aterrimus* Lehmann & Neumann(36) or *B. mesentericus niger* Lunt(37). The two types have been well differentiated by Clark & Smith(38), but pigment formation is not a stable feature of all strains. From one strain of each type variants were separated which are identical in cultural and morphological characters with the variants already described. Those of the *B. niger* strain show all degrees of pigmentation, and some fail to produce even a faint darkening of suitable media. Among the variants of the *B. aterrimus* type some produce on glucose agar a pigment which is merely grey or brown; others fail to darken glucose agar but produce on potato or corn mash a red pigment only. None of the variants of this strain lost entirely the ability to produce at least a faint pink colour on potato and mash. Further evidence of the inconstancy of the black pigments was obtained from three strains of the N.C.T.C. One is *B. niger* provided by Gorini and another is a Kral culture of the same type sent by Ford. Both are feeble pigment producers compared with recently isolated organisms. The third, a strain of *B. aterrimus* received from Ford is incapable of producing either a black or a pink colour. Since this work was done Gordon & Smith(39) have reported identical results on the loss of black pigmentation.

Conclusions. The changes in cultural character described above cover almost the entire range of variation observed among the original strains and also most of the modifications known to exist among 'potato bacilli' or the '*B. mesentericus* group'. Practically only one property in which the original strains were found to differ from each other, the ability to reduce nitrate, has not been proved to be unstable. It may therefore be concluded that unless future work reveals some important division in this group, its various members, despite the fact that they exhibit some striking cultural differences, should all be included in the species *B. subtilis*. There does not even appear to be any advantage in according varietal status to the viscous and pigmented types for their differential characters are neither sharply defined nor completely stable.

Stock cultures examined

Several type cultures were discussed in the foregoing paragraphs. A number of other named cultures, received from various laboratories, proved to be strains of *B. subtilis*. Some were obviously incorrectly named and need not be considered further. The remaining strains provide evidence concerning the synonyms of *B. subtilis* and deserve the following brief comments.

B. vulgatus (N.C.T.C. 2588), a strain provided by Ford *et al.* (16), conforms to their description of that species and furnishes proof that they used the name *B. subtilis* for a different organism (see *B. licheniformis*).

A culture of *B. leptosporus* Klein (40) which Migula sent to Kral (given to me by Dr H. J. Conn) and Hueppe's (41) original strain of *B. butyricus* (N.C.T.C. 1650) were identified as *B. subtilis*. Neide's (42) description of *B. lacticola* does not support his suggestion that *B. butyricus* is a synonym of that species.

Cultures of several of the species of *Tyrothrix* isolated by Duclaux (43) were also classified as *B. subtilis*. They, together with their numbers in the N.C.T.C., are *T. geniculatus* 2123, *T. minimus* 2122, *T. scaber* vars. A and B, 2116 and 2117, *T. tenuis* vars. A and B, 2118 and 2119. These strains were sent to the N.C.T.C. by Rosenthal who stated (44) that they are authentic cultures received by him from Fernbach. They have been found to have a lytic action on other bacteria (44), and are of historical interest owing to their association with a controversy concerning the agents responsible for the ripening of cheese (45), but their identity has received little attention previously.

Synonyms of Bacillus subtilis

Summarizing the foregoing discussion, some of the probable synonyms of *B. subtilis* are:

B. aterrimus Lehmann & Neumann (*B. mesentericus niger* Lunt)
B. butyricus Hueppe
B. globigii Migula
B. leptosporus Klein
B. levaniformans β Greig Smith
B. liodermos Flügge
B. mesentericus Trevisan (*B. mesentericus fuscus* Flügge)
B. mesentericus ruber Flügge
B. niger Migula (*B. lactis niger* Gorini)
B. panis (Vogel) Migula
B. viscogenes Hood & White
B. viscosus berolinensis Seligmann
B. vulgatus Trevisan (*B. mesentericus vulgatus* Flügge)
Bacterium mesentericum (Vogel) Migula
Bacterium panis Fuhrmann
Tyrothrix geniculatus Duclaux
Tyrothrix minimus Duclaux
Tyrothrix scaber Duclaux
Tyrothrix tenuis Duclaux

This list is certainly far from complete, but until cultures of many briefly described organisms are examined it will be difficult to extend it appreciably with any confidence.

Bacillus pumilus

Of the strains examined, eighty, including one of Gottheil's (5) original cultures of *B. pumilus* and one of Ford's (16) strains of *B. mesentericus*, have been assigned to this species. They closely resemble the variants of *B. subtilis* which produce on solid media a smooth glossy growth. Their colonies on agar are (if spreading does not occur) predominantly of the type shown in Pl. 1, fig. 5 and only exceptionally show a roughened surface. In morphology most strains cannot be differentiated from *B. subtilis*, but in a few the cells are slightly smaller, being about 0·5 μ thick after staining (Pl. 1, fig. 2). There are several distinctions from *B. subtilis*. The proteolytic action on gelatin and milk is frequently slower and nitrite is never produced from nitrate. Diastase is never secreted, a feature which is at least partly responsible for the poorer growth on potato and bread. On potato the growth is relatively thin and usually smooth (Pl. 1, fig. 15), and the medium becomes brown; certain strains may show slight wrinkling in the early stages of incubation (Pl. 1, fig. 16). The acidity produced from fermentable sugars is more persistent than is usual with *B. subtilis*, and as a consequence the growth on glucose agar is restrained. The spores of *B. pumilus* are in general less tolerant of heat, and growth occurs in a somewhat lower range of temperature than in the case of *B. subtilis*.

All strains can proliferate at 45° C., and some do so to a small extent at 50° C. At 10° C. growth becomes visible in 7–14 days. Red and black pigments and markedly viscous growths have not been detected. Ecological observations have also indicated certain differences: *B. pumilus* occurs more numerously in soil and, unlike *B. subtilis*, only rarely colonizes dairy equipment.

It may well be questioned whether these distinctions are adequate to separate two species for none is decisive except diastase production. Several writers have used nitrate reduction as a differential character, but occasional strains of otherwise typical *B. subtilis* fail to reduce nitrate. Lamanna (12) has shown that spore agglutination affords no separation, and Flynn & Rettger (30), noting the similarity of cultural variants, concluded that the two types constitute a single species. It is suggested here that the specific rank of *B. pumilus* should be continued meantime.

Chester, in contributions (21, 46) which have had considerable influence, classified Gottheil's *B. pumilus* as *B. mesentericus*, and this convention has been followed by Ford (16, 47) and others in America. It is not clear why such nomenclature was adopted, for the earlier descriptions of *B. mesentericus* by Flügge (14), Lehmann & Neumann (36), Migula (20) and others, by their references to brown rugose growths on agar and potato, strongly suggest—even when allowance is made for variations in the composition of media—that they apply to the type of organism now accepted as typical *B. subtilis*. Moreover, this conception of *B. mesentericus* is still common. Thus, whatever the future status of *B. pumilus* may be, *B. mesentericus* should be regarded as a synonym of *B. subtilis*.

A number of strains received from the N.C.T.C. proved to be of the *B. pumilus* type. Of these the following deserve mention:

B. cohaerens (N.C.T.C. 2596), a Kral culture which had been received from Ford, conforms to the description of that species given by Ford *et al.* (16). It would appear that these workers should not have accepted this strain as authentic, for Gottheil's (5) original description of *B. cohaerens* indicates that the organism belongs to the *B. carotarum* type (48).

B. globigii (N.C.T.C. 2593) had been provided by Ford who obtained it from Kral's collection. It fits Ford's (16) description and is quite different from the organisms which others have recognized as *B. globigii*.

B. dendroides (N.C.T.C. 1653), reputed to be Holzmüller's (49) original strain, has no feature incompatible with the meagre description which that species received.

Other strains of inadequately described species which were found to be of the *B. pumilus* type are *B. truffauti* Truffaut & Bezssonoff (50) (N.C.T.C. 1490), and *Leptothrix haemoglobinophila sporulans* Mackenzie (51) (N.C.T.C. 2170 and 3490).

Bacillus licheniformis

This species, of which ninety-eight strains were examined, has been the cause of much confusion for it was regarded as *B. subtilis* by Ford *et al.* (16) in their important contribution to the taxonomy of *Bacillus*, and it is apparently still merged with that species by Bergey (52). Lamanna (12, 6) finds that its spores differ antigenically from those of *B. subtilis* as shown by agglutination and precipitation tests. In cultural character it exhibits comparatively little variation, and it may be distinguished from *B. subtilis* by the following features:

Colonies. Surface colonies on agar may be like any of those of *B. subtilis* but are frequently distinguishable (Pl. 1, fig. 6) by producing hair-like or woolly outgrowths, or by being firmly attached to the medium, or by forming (especially on alkaline glucose agar) vesicles or lobes of a mucoid character. Deep colonies often show a characteristically diffuse margin owing to penetration of the agar (Pl. 1, fig. 7).

Agar slope. On ordinary agar the growth is frequently mucoid in the early stages, but it soon becomes dull and rough or wrinkled. It tends to spread if the medium is moist, and it may be strongly attached to the agar. The mucoid and adherent properties are promoted by an alkaline reaction. Ciliate or tangled outgrowths (Pl. 1, fig. 11) generally develop at the edge (a diagnostic character). Later the growth becomes greyish white and more refractile. A rugose pellicle generally forms on condensation water. In variants which can be separated from typical strains the growth may exhibit a ground-glass appearance or remain relatively thin and grey.

On glucose agar the growth has a strong tendency to be mucoid in the early stages and wrinkled later, and it usually becomes slightly brownish. The occurrence of mucoid vesicles in a 'dry' rugose growth is, when observed, diagnostic.

Glucose agar stab. A luxuriant growth is produced to the extremity of the stab (Pl. 1, fig. 8*b*) and slight gas formation may be detected if the medium has been stored for some time and is not heated before inoculation. Surface growth usually becomes rugose and brownish; it may contain glistening vesicles. Most strains form a disk of reddish pigmentation in the agar about 1 cm. below the surface.

Gelatin stab. Liquefaction is slow, the liquid zone being never more than saucer-shaped on the seventh day at 22° C. (Pl. 1, fig. 9*b*). The stab is frequently villous and the surface growth may be reddish.

Potato. In many strains the growth is at first mucoid, but it soon appears to become dry and shrivelled and forms brownish wrinkles among which blister-like swellings may persist for a time (Pl. 1, fig. 17). The growth of other strains may have a dry appearance from the beginning and wrinkles may not form (Pl. 1, fig. 18). Certain variants produce a more refractile smooth growth. Variations in the medium affect the results appreciably. Many strains produce a reddish pigment.

Milk. The reaction becomes slightly acid and at 7 days (37° C.) there is usually a smooth or granular curd and a turbid whey (Pl. 1, fig. 10*b*). Digestion of the curd generally proceeds only slowly, but certain strains in certain samples of milk produce almost complete clearing within 7 days. Litmus in milk is usually reduced at an early stage. Cultures placed at room temperature generally become alkaline, the change starting at the surface. Most strains form a pellicle.

Diastase. Only one strain of this series fails to secrete diastase.

CO_2 *production.* Traces of visible gas may occasionally be formed from glucose in media of the usual composition. Free gas is regularly produced at 37° C. in Gibson & Abdel-Malek's (53) test in which a high concentration of glucose and a strongly buffered medium are used.* *B. subtilis* shows no gas formation in this test.

Nitrate. Reduced to nitrite. In a rich medium (e.g. 5% peptone + 5% glucose + 1% KNO_3), made alkaline after sterilization, gas is produced vigorously. Much of the gas is CO_2 but a proportion is not absorbed by alkali, indicating that denitrification occurs.

Oxygen requirement. Anaerobic growth occurs readily in glucose-containing media, but aerobically the growth is much more vigorous.

Temperature requirement. Growth occurs rapidly at 50° C. and many strains proliferate at 55° C. Below 20° C. growth is slow; the minimum temperature for the production from small inocula of a visible growth within 4 weeks is in the region of 12–15° C.

Pigments. Black pigments have not been encountered; the red pigment is produced by a greater proportion of strains than in *B. subtilis*. Otherwise there is no distinction in pigment formation.

Nomenclature

Among the strains which were examined are several received from other laboratories. They include cultures labelled *B. globigii*, *B. subtilis*, *B. mesentericus*, *B. vulgatus*, *B. panis*, *B. mycoides*, *Leptothrix* and an original strain (2120 in the N.C.T.C.) of *Tyrothrix filiformis* Duclaux (43). The cultures received as *B. mycoides* had possibly been wrongly identified owing to a superficial resemblance of the colonies of the two types on certain media. The other cultures show that the species has sometimes been identified

* To litmus milk add 5% glucose (from a 50% solution) and melted nutrient agar in the approximate proportion of one part of agar to four of milk. Inoculate while still liquid, chill the tube, gently run in nutrient agar so that it forms a solid layer 2–3 cm. deep above the milk and incubate.

as *B. subtilis* or given a synonymous designation. Two of the cultures labelled *B. subtilis* (possibly descendants of the same parent strain) were supplied by Ford. Both conform to his descriptions (16, 47), thus establishing the fact that he regarded organisms of this type as *B. subtilis*. His attitude is perhaps to be explained by the fact that he based his identification on a culture received from Chester, apparently believing that it was identical with Gottheil's (5) *B. subtilis*. Chester's paper (21) suggests, however, that he may have included two types in the species *B. subtilis* and, if he did so, this might account for the culture he sent to Ford.

Of the cultures received, only those labelled *B. globigii* might claim to be correctly named, but the validity of this designation is also questionable. Bacteriological literature contains numerous descriptions which evidently apply to the species, and the following list gives in chronological order specific designations which appear to have been given to it:

1898. *Clostridium licheniforme* Weigmann (54).
1900. *Clostridium gelatinosum* Laxa (55).
1902. *Bacillus levaniformans* α Greig Smith (19).
1904. *Bacillus parvus* Neide (42).
1905. *Semiclostridium commune* Maassen (56).
1907. *Bacillus mesentericus ruber* as described by Gruber (57).
1916. *Bacillus subtilis* Ford *et al.* (16).
1925. *Bacillus globigii* Migula, as described by Fred, Peterson & Carroll (58).

Perhaps the most frequently used name has been *B. mesentericus ruber* Flügge (14), but prior to the appearance of Gruber's paper (57) there seems to have been no description using that designation which applies definitely to this species. Most of the earlier descriptions appear to deal with strains of *B. subtilis* which produce a red pigment on potato. Undoubtedly the term 'red potato bacillus' has been used for strains of both species.

There has been little unanimity on the identity of *B. globigii* which is often considered to be a synonym of *B. mesentericus ruber*. Migula's original characterization (20) suggests that it refers to *B. subtilis* and, as shown in an earlier paragraph, Ford *et al.* (16) used this name for a strain of *B. pumilus*. It would appear that the term *B. globigii* cannot be proved to have been applied to the species described here before the publication of the paper by Fred *et al.* (58). The description they give is inconclusive, but one of their cultures was examined by Cunningham (59) who proved its identity.

In view of the foregoing discussion there can be little reason for ignoring the first name known to be associated with an adequate account of the species and, as proposed in a preliminary communication (7), the correct designation is the new combination *Bacillus licheniformis* (Weigmann). Some years ago Cunningham (59) also drew attention to the priority of Weigmann's contribution.

Summary

A group of species, comprising *Bacillus subtilis* and related organisms, may be differentiated from other members of the genus *Bacillus* by (*a*) morphology and (*b*) the production of acid and acetylmethylcarbinol from glucose. The group may be divided into three species:

(1) *B. subtilis* Cohn *emend.* Prazmowski, the synonyms of which include *B. aterrimus*, *B. globigii*, *B. mesentericus*, *B. niger*, *B. panis*, and *B. vulgatus*.

(2) *B. pumilus* Gottheil, which resembles strains of (1) that produce a smooth glossy growth. It differs in failing to secrete diastase and in other features, but its separation as a distinct species is still an open question. It is sometimes identified as *B. mesentericus*.

(3) *B. licheniformis* (Weigmann) Gibson, the synonyms of which include *B. subtilis* (as used by Ford), *B. globigii* and *B. mesentericus ruber*. This species may be differentiated from (1) and (2) by its cultural characters, slow proteolytic action, vigorous CO_2 production and ability to grow anaerobically.

The writer desires to thank all those who have given him cultures, and especially Dr R. St John-Brooks, who provided many interesting strains. Thanks are also due to Dr L. E. Topping, who isolated some of the organisms and confirmed the conclusions of this investigation.

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EXPLANATION OF PLATE

Fig. 1. *B. subtilis*. Large-cell type. 24 hr., 30° C. Aq. fuchsin. ×1000.
Fig. 2. *B. pumilus*. 24 hr., 30° C. Aq. fuchsin. ×1000.
Fig. 3. Colony of *B. subtilis*. Rugose type. Ordinary agar 3 days, 37° C. ×1.
Fig. 4. Colony of *B. subtilis*. Type with dust-like surface. Ordinary agar 1 day, 30° C. Oblique transmitted light. ×1.
Fig. 5. Colony of *B. subtilis*. Smooth glossy type. Ordinary agar 2 days, 37° C. ×1.
Fig. 6. Colony of *B. licheniformis*. Ordinary agar 1 day, 37° C. ×1.
Fig. 7. Deep colony of *B. licheniformis*. Ordinary agar 2 days, 37° C. Oblique transmitted light. ×1.
Fig. 8. Glucose agar stab cultures. 7 days, 37° C. (*a*) *B. subtilis*; (*b*) *B. licheniformis*.
Fig. 9. Gelatin stab cultures. 7 days, 22° C. (*a*) *B. subtilis* (type producing pellicle and moderate turbidity); (*b*) *B. licheniformis*.
Fig. 10. Brom cresol purple milk cultures. 7 days, 37° C. (*a*) *B. subtilis* (completely cleared and alkaline); (*b*) *B. licheniformis* (curd and whey; slightly acid).
Fig. 11. Slope culture of *B. licheniformis*. Ordinary agar 3 days, 37° C. Transmitted light.
Figs. 12–14. Potato cultures of *B. subtilis*.
Figs. 15, 16. Potato cultures of *B. pumilus*.
Figs. 17, 18. Potato cultures of *B. licheniformis*.

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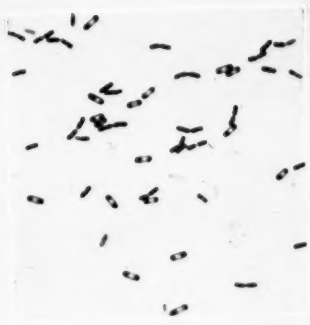

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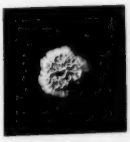

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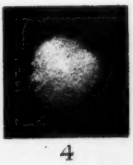

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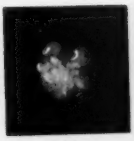

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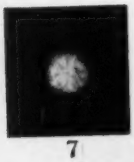

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307. THE OCCURRENCE IN WINTER OF MILK WITH A LOW CONTENT OF SOLIDS-NOT-FAT

By S. J. ROWLAND, *National Institute for Research in Dairying, University of Reading*

(With 1 Figure)

The solids-not-fat content of the milk produced in some parts of the country has for the last two or three years fallen to a very low level during the winter months, that is at a time of the year when, judging from pre-war experience, the solids-not-fat content would be expected to be high. Early in March 1942 an investigation was started of the solids-not-fat content of the milk of a large number of the individual herds in a well-known dairying area where the general level of solids-not-fat was known to be low. The investigation was continued until May of that year, resumed in January 1943, and continued until the following June. The data which have been obtained in this particular area are briefly described herewith, for they provide a good example of the occurrence of low solids-not-fat in winter and indicate its probable cause.

Investigation in 1942

During March and early April 447 samples of milk from different producers were collected at random on delivery at eleven creameries in the area, approximately the same number of samples being taken at each creamery. The samples were sent to this Institute, where their total solids content was determined gravimetrically by evaporation in the steam oven, using the Golding (1) technique, and their fat content was determined by the Gerber method (2). Freezing-point determinations, details of which are given by Aschaffenburg, Temple & Veinoglou (3) as part of a general survey of the extent to which added water is present in commercial milk supplies, showed that twenty-three of the milk samples contained some added water, and these samples were therefore excluded from further consideration. The composition of the milk of each of the eleven creameries was closely similar, and the distribution of the solids-not-fat percentage of the 424 genuine samples is summarized in the first part of Table 1. The mean solids-not-fat content was only 8·51%, and 47·6% of the samples were below the presumptive legal standard of 8·50%.

During May a further 240 samples were collected at random from five of the creameries. Freezing-point determinations showed that ten samples contained added water. The solids-not-fat distribution of the 230 genuine samples is shown in the second part of Table 1. The mean solids-not-fat content had improved to 8·68%, with 25·2% of the samples below the standard.

Investigation in 1943

Of the eleven creameries at which samples were taken in 1942, two typical creameries were selected for study in 1943. These will be referred to as creamery A and creamery B. The milk of thirty-six representative producers at creamery A, and of seventy-two at creamery B, was sampled fortnightly from January onwards, the milk of the same pro-

ducers being sampled on each occasion. This procedure was adopted in order to get reliable data on any change in the solids-not-fat content of the milk with time of year. The samples were tested for total solids and fat in the same manner as the samples taken in 1942. In April the milk of one producer at creamery A and fourteen producers

Table 1. *Distribution of solids-not-fat in the milk samples taken in* 1942

Solids-not-fat %	Samples taken during March and April		Samples taken during May	
	No. of samples	% of samples	No. of samples	% of samples
9·30–9·39	—	—	3	1·3
9·20–9·29	1	0·2	2	0·9
9·10–9·19	2	0·5	3	1·3
9·00–9·09	7	1·7	10	4·3
8·90–8·99	23	5·4	27	11·7
8·80–8·89	24	5·7	30	13·0
8·70–8·79	40	9·4	38	16·6
8·60–8·69	62	14·6	39	17·0
8·50–8·59	63	14·9	20	8·7
8·40–8·49	51	12·0	27	11·7
8·30–8·39	69	16·3	14	6·1
8·20–8·29	28	6·6	14	6·1
8·10–8·19	27	6·4	3	1·3
8·00–8·09	17	4·0	—	—
7·90–7·99	4	0·9	—	—
7·80–7·89	2	0·5	—	—
7·70–7·79	2	0·5	—	—
7·60–7·69	2	0·5	—	—
Total	424	100·0	230	100·0
Mean S.N.F. %	8·51		8·68	

Table 2. *Distribution of solids-not-fat in the milk samples taken at creamery 'A' in* 1943

Solids-not-fat %	No. of samples										
	18 Jan.	1 Feb.	15 Feb.	1 Mar.	15 Mar.	29 Mar.	12 Apr.	26 Apr.	10 May	24 May	7 June
>9·10	—	—	—	—	—	—	—	—	3	4	3
9·00–9·09	—	—	—	—	—	—	—	1	2	4	6
8·90–8·99	1	—	—	—	—	—	—	5	8	4	5
8·80–8·89	1	—	2	—	—	3	—	2	8	6	4
8·70–8·79	2	4	2	1	1	2	3	5	6	6	6
8·60–8·69	3	4	5	1	6	7	3	8	3	4	4
8·50–8·59	9	8	8	3	8	11	12	4	1	4	5
8·40–8·49	10	7	8	7	7	4	8	3	1	2	1
8·30–8·39	6	7	4	12	3	3	3	2	1	—	1
8·20–8·29	2	2	2	6	5	2	3	2	—	—	—
8·10–8·19	—	2	2	2	1	3	2	1	—	—	—
8·00–8·09	—	—	—	1	2	—	—	—	—	—	—
<8·00	1	1	2	2	2	—	1	—	—	—	—
Total	35	35	35	35	35	35	35	33	33	34	35
Mean S.N.F. %	8·49	8·46	8·46	8·33	8·40	8·53	8·46	8·65	8·84	8·82	8·81
Standard deviation	0·18	0·18	0·23	0·18	0·22	0·19	0·16	0·22	0·18	0·21	0·21
Percentage of samples below 8·50% S.N.F.	54	54	51	86	57	34	49	24	6	6	6
Mean fat %	3·59	3·55	3·49	3·60	3·57	3·37	3·40	3·31	3·43	3·32	3·26

at creamery B ceased to be available. Results from January until June were obtained for thirty-five of the producers at creamery A and fifty-eight at creamery B. These results are summarized in Tables 2 and 3. In illustration of the trend of the results for individual producers the full series of results for creamery A is given in Table 4. The lengthy series for creamery B was closely similar.

Table 3. *Distribution of solids-not-fat in the milk samples taken at creamery 'B' in* 1943

Solids-not-fat %	No. of samples									
	11 Jan.	25 Jan.	8 Feb.	22 Feb.	8 Mar.	22 Mar.	5 Apr.	19 Apr.	31 May	14 June
>9·10	—	1	2	—	—	—	—	—	2	5
9·00–9·09	—	1	1	—	—	1	1	—	6	4
8·90–8·99	5	4	—	2	—	1	—	—	7	17
8·80–8·89	3	1	2	1	4	1	2	1	22	16
8·70–8·79	5	8	8	1	2	2	3	8	9	9
8·60–8·69	8	10	17	2	2	2	5	10	7	2
8·50–8·59	18	8	13	5	10	8	10	12	2	3
8·40–8·49	7	12	10	10	10	12	12	12	1	1
8·30–8·39	9	5	3	18	11	13	11	8	1	—
8·20–8·29	1	2	1	11	10	10	6	3	—	—
8·10–8·19	2	3	1	3	4	4	1	4	—	—
8·00–8·09	—	1	—	3	3	3	3	—	—	—
<8·00	—	2	—	2	2	—	2	—	—	—
Total	58	58	58	58	58	57	56	58	57	57
Mean S.N.F. %	8·57	8·55	8·60	8·36	8·40	8·40	8·44	8·51	8·83	8·88
Standard deviation	0·19	0·25	0·19	0·22	0·22	0·20	0·21	0·18	0·17	0·16
Percentage of samples below 8·50% S.N.F.	33	43	26	81	69	74	63	47	4	2
Mean fat %	3·62	3·58	3·54	3·56	3·53	3·52	3·26	3·34	3·31	3·32

Table 4. *Percentage of solids-not-fat in the milk samples taken at creamery 'A' in* 1943

Producer no.	18 Jan.	1 Feb.	15 Feb.	1 Mar.	15 Mar.	29 Mar.	12 Apr.	26 Apr.	10 May	24 May	7 June
1	8·34	8·50	8·28	8·37	8·42	8·31	8·26	8·28	8·49	9·04	8·72
2	8·75	8·62	8·45	8·45	8·50	8·55	8·55	8·79	8·91	8·73	8·83
3	8·73	8·61	8·51	8·39	8·44	8·69	8·60	8·67	8·77	9·03	9·00
4	8·54	8·19	7·99	8·00	8·03	8·15	8·13	8·41	8·66	8·61	8·54
5	8·21	8·35	8·40	8·46	8·68	8·60	8·55	8·52	8·90	8·74	8·75
6	8·23	8·26	8·34	8·51	8·61	8·67	8·50	8·92	9·12	8·99	8·91
7	7·93	7·88	7·73	7·60	7·76	8·13	7·85	8·33	8·60	8·58	8·58
8	8·32	8·25	8·31	8·36	8·60	8·68	8·38	—	8·93	8·78	8·90
9	8·49	8·55	8·42	8·49	8·41	8·35	8·49	8·86	8·80	8·44	8·34
10	8·64	8·57	8·51	8·42	8·61	8·76	8·70	8·68	8·82	8·79	9·09
11	8·36	8·32	8·17	7·81	7·94	8·28	8·20	8·40	8·83	8·68	8·70
12	8·55	8·38	8·42	8·26	8·44	8·51	8·46	8·92	—	8·90	8·76
13	8·59	8·42	8·47	8·34	8·35	8·67	8·52	8·66	8·74	8·69	8·65
14	8·46	8·36	8·37	8·20	8·20	8·48	8·37	8·63	8·68	8·56	8·58
15	8·47	8·41	8·53	8·29	8·28	8·53	8·38	—	9·04	8·78	8·74
16	8·38	8·74	8·65	8·21	8·43	8·48	8·42	8·95	8·99	8·87	8·87
17	8·98	8·70	8·82	8·63	8·66	8·84	8·57	8·78	8·99	8·89	8·65
18	8·46	8·49	8·68	8·39	8·43	8·58	8·55	8·98	9·22	9·20	9·18
19	8·83	8·57	8·65	8·37	8·19	8·17	8·23	8·28	8·58	8·44	8·47
20	8·53	8·73	8·72	8·53	8·53	8·61	8·52	8·71	8·86	8·68	8·69
21	8·58	8·35	8·48	8·27	8·06	8·57	8·45	8·66	8·75	8·55	8·58
23	8·46	8·17	8·51	8·29	8·20	8·50	8·45	8·56	8·80	8·81	8·95
24	8·51	8·47	8·35	8·35	8·72	8·76	8·79	8·90	9·18	9·21	9·16
25	8·61	8·59	8·66	8·78	8·53	8·88	8·71	8·64	9·01	9·11	9·11
26	8·41	8·32	8·17	8·37	8·59	8·50	8·59	9·04	8·86	9·14	8·84
27	8·46	8·50	8·44	8·30	8·53	8·57	8·50	8·68	8·74	9·01	9·01
28	8·41	8·32	8·75	8·46	8·53	8·49	8·61	8·56	8·72	9·01	8·96
29	8·33	8·68	8·55	8·13	8·20	8·28	8·15	8·18	8·30	8·50	8·53
30	8·52	8·42	8·27	8·17	8·27	8·34	8·40	8·45	8·87	8·90	8·81
31	8·52	8·70	8·63	8·41	8·40	8·50	8·52	8·56	8·77	8·85	9·02
32	8·65	8·50	8·57	8·56	8·57	8·67	8·49	8·72	8·96	8·86	8·62
33	8·53	8·67	8·87	8·46	8·56	8·56	8·56	8·64	—	8·76	8·78
34	8·46	8·47	8·55	8·38	8·61	8·84	8·68	8·73	8·89	—	8·91
35	8·45	8·52	8·48	8·39	8·36	8·51	8·52	8·85	8·91	8·90	9·00
36	8·39	8·45	8·55	8·30	8·37	8·42	8·41	8·39	8·98	8·89	9·04

Determinations of freezing-point were made on one set of samples from each creamery. The samples from creamery A were tested on 15 March and all were found to be genuine. Thirty-six of the samples from creamery B, taken at random, were tested on 8 March, and one sample was found to contain some added water. Although a few of the samples for which the results are given in Tables 2 and 3 may have contained some added water, the effect on the results as a whole may be taken as negligible.

The solids-not-fat content of the samples from creamery A was extremely low from 18 January until 12 April; the mean for all the samples taken during this period was only 8·45%, and 55% of the samples were below the presumptive legal standard of

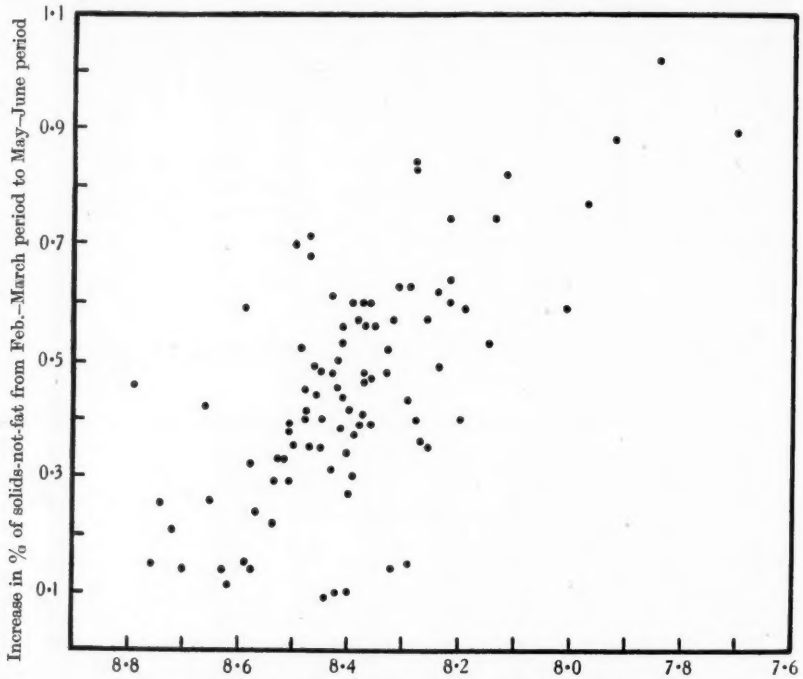

Fig. 1. The relationship between the increase in solids-not-fat and the winter level of solids-not-fat.

8·50%. On only one occasion during this period did the mean solids-not-fat content of the samples exceed this standard—on 29 March when it was 8·53%. The minimum mean solids-not-fat content, 8·33%, occurred on 1 March, 86% of the samples being below the presumptive standard.

On 26 April the mean solids-not-fat content had improved to 8·65%, and on 10 May to a normal figure of 8·84%. Thus in four weeks from 12 April to 10 May the mean percentage of solids-not-fat increased by 0·38, and the percentage of samples below the presumptive standard had decreased from 49 to 6. The high level of solids-not-fat reached on 10 May was maintained.

The results for the samples from creamery B were essentially the same as those from creamery A; the mean solids-not-fat content of all the samples taken from 11 January

to 19 April was only 8·48%, 54% of these samples being below the presumptive standard. The minimum mean solids-not-fat content was 8·36 on 22 February when 81% of the samples were below the standard. On 31 May the mean solids-not-fat content had increased to a normal figure of 8·83% and on 14 June to 8·88%, when only one sample out of fifty-seven was below the standard. Unfortunately, owing to unforeseen circumstances, data are not available for 3 and 17 May.

The data presented have two noteworthy features; the persistently low solids-not-fat content of the milk until about the middle of April, and the rapid increase in the solids-not-fat content to normal levels in May. The marked difference between the solids-not-fat content of the milk produced in the winter and that of the milk produced in spring and early summer is, however, best revealed by an examination of the relevant data for each producer separately. For each of the producers at creamery A, the mean solids-not-fat content of the three samples taken on 10 May, 24 May and 7 June has been compared with that of the three samples taken on 15 February, 1 March and 15 March. For each of the producers at creamery B, the mean of the two samples taken on 31 May and 14 June has been compared with the mean of the three samples taken on 22 February, 8 March and 22 March. The solids-not-fat content of the milk of each of the total of ninety-three producers was, without exception, greater in the May–June period than in the winter period, the mean increase in the solids-not-fat percentage being 0·45. The distribution of the increases is summarized in Table 5, and the relationship between the increase in solids-not-fat and the winter level of solids-not-fat is shown in Fig. 1. In the milk of 67% of the producers the increase was between 0·30 and 0·65%.

Table 5. *Increases in percentage of solids-not-fat from February–March period to May–June period*

	No. of producers		
Increase in % solids-not-fat	Creamery A	Creamery B	Creameries A and B
1·00–1·09	—	1	1
0·90–0·99	—	—	—
0·80–0·89	1	4	5
0·70–0·79	3	2	5
0·60–0·69	1	9	10
0·50–0·59	7	7	14
0·40–0·49	7	15	22
0·30–0·39	8	9	17
0·20–0·29	2	6	8
0·10–0·19	5	5	10
0·00–0·09	1	—	1
Total	35	58	93
Mean increase	0·42	0·47	0·45

DISCUSSION

The occurrence during the past two winters of genuine milk with a low content of solids-not-fat is by no means confined to one county or district. Evidence of it (not presented in this paper) has been obtained from at least four districts in widely separate areas of England. Such a widespread phenomenon has important legal and nutritional bearings, and it is obviously important to establish its cause and evolve remedial measures. The creamery or dairyman may refuse to accept milk below the presumptive standard in solids-not-fat as not being of the quality demanded under the terms of the producer's contract, with consequent financial loss to the producer and wastage of milk.

Alternatively the producer may be informed that his milk is deficient in solids-not-fat and be requested to give the matter his attention—a form of words which he is liable to interpret as an implication that his milk contains added water. As his milk is unadulterated, the producer is naturally anxious to know in what way the solids-not-fat content of his milk can be improved. From the strictly legal point of view the producer is open to prosecution under the Sale of Milk Regulations whereby 'if a sample of milk contains less than 8·5% of milk solids other than milk fat it should be presumed, until the contrary is proved, that the milk is not genuine....' The main significance, however, lies in the nutritional aspect. The consumer who buys such milk is receiving a foodstuff whose inferiority to the normal milk he believes he is purchasing is roughly proportional to its deficit in solids-not-fat.

On the basis of the data obtained during the investigation described above it seems likely that the low levels of solids-not-fat which occurred during the early months of the year were due to a low plane of nutrition of the dairy herds during that period. At least, no other explanation can at present be given of the rapid and general increase which was observed in the solids-not-fat content of the milk in late April and early May, that is at the time when the herds had access to an ample supply of young grass of high nutritive value, and of the tendency, as shown in Fig. 1, for the lowest winter levels of solids-not-fat to be associated with the largest increases in solids-not-fat in spring.

Riddet, Campbell, McDowall & Cox(4) have recently shown that underfeeding of the cow causes an appreciable reduction in the solids-not-fat content of her milk, and the winter level of feeding of many of the dairy herds in the area with which the present investigation deals was certainly lower under the existing war-time conditions than it had been before the war. This may be attributed largely to inexperience of the production and feeding of the arable crops on which increased reliance has to be placed in view of the reduced supply of concentrates and the transition from grassland to arable farming. On farms where the solids-not-fat content of the milk produced has been unusually low during recent winters it therefore appears that increased attention should be given to the adequate provision of suitable home-grown feeding stuffs.

Summary

Data are given which show that the solids-not-fat content of the milk produced in a certain dairying area in the early months of 1942 and 1943 was unusually low, a high proportion of the individual herds yielding milk containing less than the presumptive standard of 8·5% solids-not-fat. The freezing-point depression of this milk was normal. The solids-not-fat content showed substantial improvement in May. It is suggested that the low level of solids-not-fat was due to a low plane of nutrition of the cow.

The author desires to thank Prof. H. D. Kay for his constant interest and support; also Dr R. Aschaffenburg and Dr A. L. Provan (Harper Adams Agricultural College) for their co-operation.

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637.127.6:532.77

308. THE FREEZING-POINT OF MILK

II. THE INFLUENCE OF VARIOUS FACTORS AND THEIR BEARING ON THE DETECTION OF ADDED WATER

By R. ASCHAFFENBURG and B. C. VEINOGLOU
National Institute for Research in Dairying, University of Reading

(With 6 Figures)

Examination of the data given in the published investigations on the freezing-point depression (Δ) of cow's milk reveals a lack of uniformity in average values, range of natural variation and shapes of distribution curves.* Some of these variations are not surprising: different types of apparatus and thermometer have been used; in some cases arbitrary corrections intended to disclose the 'true' freezing-point of milk have been applied whilst in others they have been omitted.† Among results presented there have been some which make one doubt whether the samples tested consisted of genuine milk; other results suggest that bacterial action had changed the osmotic condition of the milk before the Δ values were determined. Conclusions have been based, in some investigations, on too few findings, or there has been no discrimination between results obtained from individual cows and those given by the mixed milk of a herd.

Even when these causes of irregularities are taken into consideration, there still remain discrepancies in the published data which are less easily explicable. Such discrepancies are to be expected if the freezing-point of milk is more dependent on environmental factors than some have believed. Possible periodic changes in the osmotic behaviour of milk are more likely to be revealed where studies, instead of being confined to odd single samples of the milk of different animals or different groups of animals, are based on the repeated examination of milk from the same individual animals, or the same herds, over a period of time. Results based on repeated examination of the milk from the same cow have been presented by Bauer(5), Abati & Sohn(6), and Aschaffenburg & Temple(7). The last-mentioned workers confirmed an observation made some years ago by Buchanan & Lowman(8) that the Δ values during the spring months may be appreciably lower than those obtained at other seasons.

A more systematic research into the extent of seasonal changes of the freezing-point depression forms part of the present investigation. Another part deals with the disputed question of the difference in Δ between morning and evening milk, a subject to which much attention has been paid, but on which opinion is divided. Some authors(6, 9, 10) have stated that no significant difference exists, others(1, 11, 12) report the Δ of morning milk to be higher than that of evening milk, whereas still others(4, 7, 13) report the opposite. In the present study an attempt has been made to clarify this contradictory

* For summaries of the literature see Refs. 1–4.

† Hortvet(2) has shown that for all practical purposes such corrections are unnecessary, and that by the use of a simple standardized technique strictly comparable results may be obtained. Unfortunately, his principles have not been adopted by all investigators.

evidence. The extensive experimental data have also been used to re-examine (*a*) the influence of other factors, such as breed, age and state of lactation of the cow on the Δ of milk, and (*b*) the correlation between Δ and milk yield, and between Δ and solids-not-fat content. The applicability of the freezing-point test to the detection of added water in milk is then reassessed in the light of the new findings reported in the first part of the paper.

Experimental

For the determination of the freezing-point depressions an improved model of the apparatus described by Temple (14) was used, an apparatus considerably more economical of time than that of Hortvet. Duplicate tests differed, in general, by no more than 0·001° C., and, confirming Temple's observation, agreed closely with those obtained with the Hortvet apparatus.

All milk samples were taken from individual cows of the Institute herd. Morning and evening milk was collected separately and immediately put into cold store. All determinations were carried out within 24 hr. of milking (mostly within a much shorter period), and it was found that with immediate refrigeration no detectable increase in Δ due to acidity development occurred within this period.

The milk from all the Shorthorn cows in the herd (usually between thirty and forty animals) was tested at approximately fortnightly intervals over a period of 16 months (17 April 1941 to 19 August 1942). These data will be referred to as the 'fortnightly sampling'.

To obtain more information about the variations occurring within the fortnightly periods, a small number of animals was selected for more frequent sampling. A group of six cows was originally chosen and gradually replaced by others as their lactations came to an end. On the average, sampling of the selected cows was carried out at intervals of approximately 3 days. The results will be referred to as the 'bi-weekly sampling'.

Four Guernsey cows belonging to the Institute herd were also sampled over a period in order to obtain an indication of the possible effect of breed on Δ.

Results

During the investigation freezing-point determinations were carried out on a total of 3831 milk samples, consisting of (*a*) fortnightly sampling, 1087 of morning and 1051 of evening milk, (*b*) bi-weekly sampling, morning milk 665 samples, evening milk 632 samples, (*c*) sampling of Guernsey cows, 198 morning samples and 198 evening samples.

The results of the main experiment are summarized in Figs. 1–3.* Fig. 1*b* shows the average Δ values for each fortnightly sampling. The full data for each of the selected cows and the mean Δ values for each date of sampling are given in Figs. 2 and 3. These data are further summarized in Fig. 1*a* showing the average Δ values for the group of selected animals for fortnightly intervals in between the fortnightly sampling of the whole herd.

A. *The effect of season*

For both the fortnightly sampling and the bi-weekly sampling the general mean for the freezing-point depression was found to be 0·548° C. Fig. 1 *a*, *b* shows that the fortnightly averages differ to a varying degree from this general mean. An *analysis of*

* For the results in full see B. C. Veinoglou, Thesis, University of Reading, 1943.

Δ (°C.) 0·560

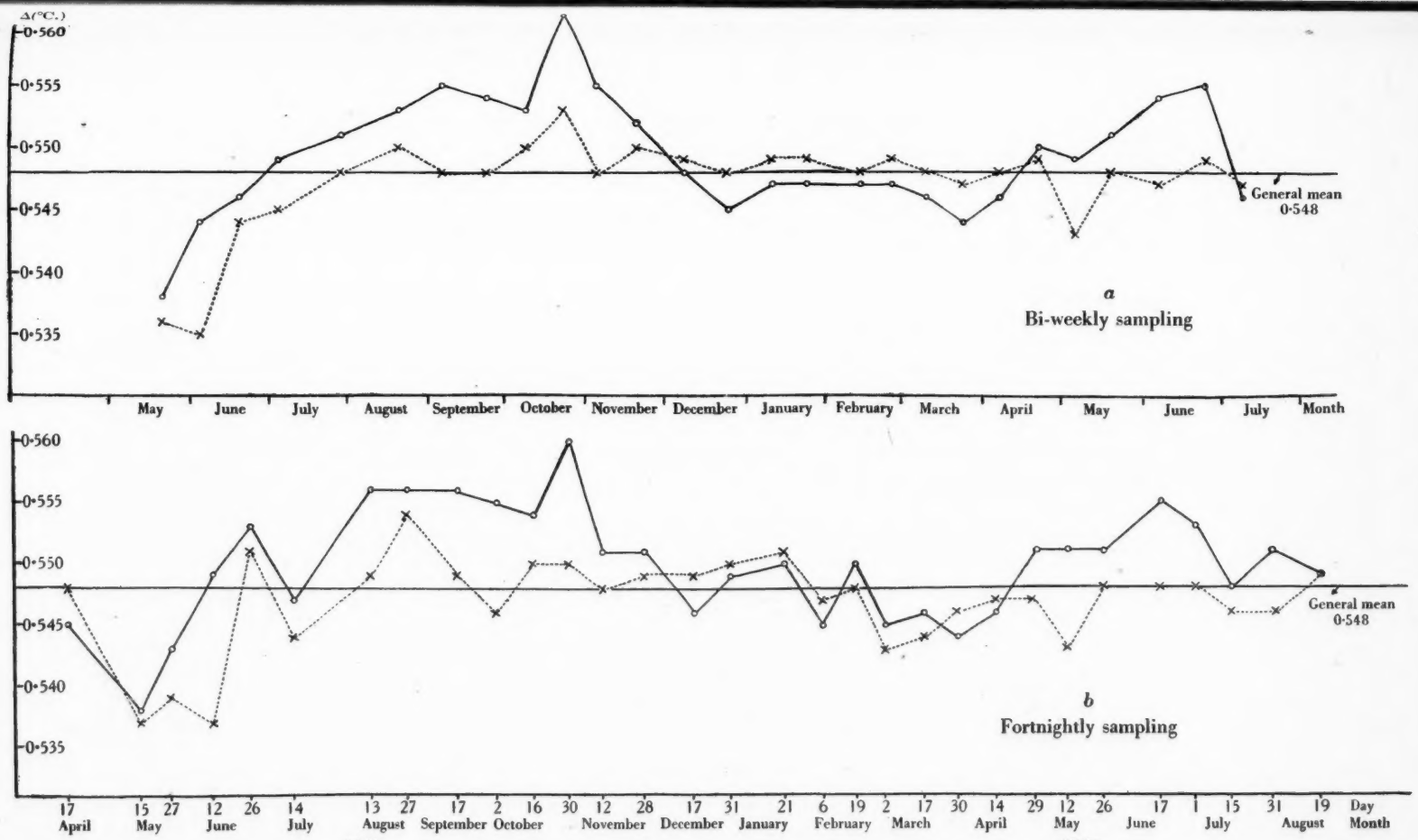

Fig. 1. × - - - × Morning milk. ○——○ Evening milk.

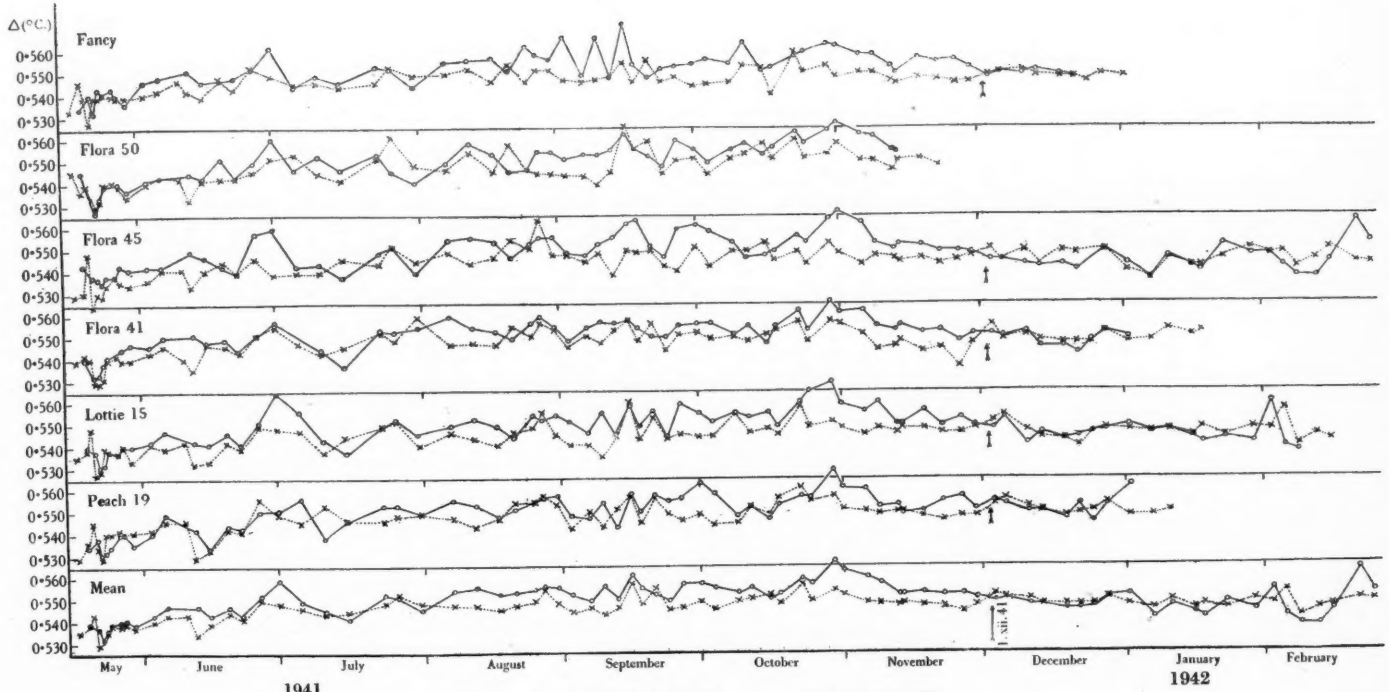

Fig. 2. × - - - × Morning milk. ○——○ Evening milk.

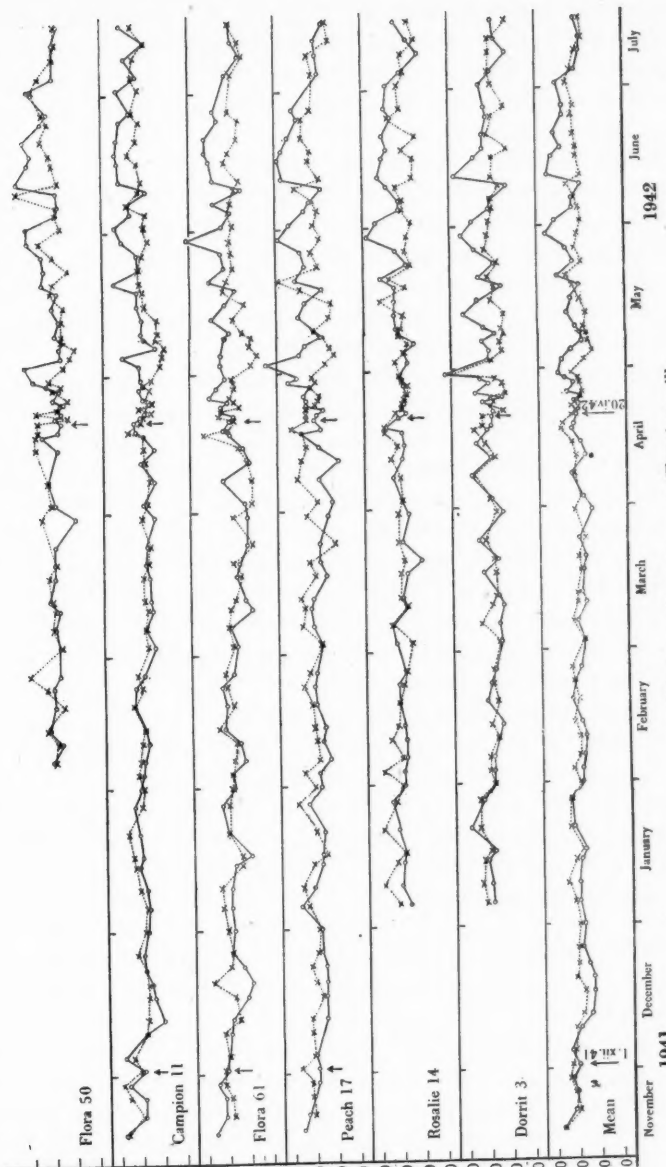

Fig. 3. × - - - × Morning milk. ○——○ Evening milk.

variance showed that any deviation of the fortnightly mean equal to or exceeding 0·002° C. must be regarded as significant on a 5% level of *P*. On the whole, the average Δ values for evening milk show a greater variability than those for morning milk. The maximum difference between the highest and lowest fortnightly average value amounted to 0·017° C. for morning milk, and to 0·022° C. for evening milk. When it is considered that we are dealing with the calculated average Δ values for milk of some thirty to forty cows, these differences are unexpectedly large.

Fig. 1 *a*, *b* shows the same general trends, a marked tendency to low Δ values in the spring of 1941, followed by a predominance of high Δ values for evening milk during the summer and autumn. Variations are far less pronounced during the winter 1941–2 and the following spring, whilst a tendency towards high Δ values with evening milk appears again in the summer of 1942. Figs. 2 and 3 support this evidence, and show, in particular, that such trends in Δ as do appear, are of a markedly general character, influencing all the cows in a similar way at the same time.

The data obtained during the spring of 1941 confirm the observation of Buchanan & Lowman (8) and of Aschaffenburg & Temple (7) that unusually low Δ values may occur during this time of year. No evidence of such a 'spring effect' was, however, observed during the second year of the present investigation. From this it can be concluded that the effect is due not to the season as such but to a factor connected with it. It has been suggested (7, 8) that the effect is bound up with the change of feeding occurring when the cattle are first turned in to fresh grass. It seems likely that the sudden change-over to a most palatable feeding stuff of a very different type from that used indoors causes a disturbance in the animal sufficient to produce a small alteration in its osmotic balance.

This suggestion is substantiated by the findings of the present investigation: during the spring of 1941 grass was satisfactory and of good quality, resulting in a marked fall in Δ values, whereas in the following spring conditions were most unsatisfactory with little growth of grass, resulting in a complete absence of the phenomenon.

It is difficult to explain the high Δ values of evening milk observed during the summer and autumn of 1941, but it seems possible that these are also bound up with the changes in the nutritional value of the grass which take place as the season advances. The persistence of these high figures—from August to November 1941—makes it unlikely that their cause is merely meteorological.

B. *Differences between the freezing-point depressions of morning and evening milk*

Figs. 1–3 show that the differences between morning and evening milk follow certain systematic trends.

Figs. 2 and 3 show that on a given day the differences are frequently, though not always, of the same sign for all or most of the cows. This was even more marked with the data for the fortnightly sampling of the whole herd which for lack of space cannot be given in full. Thus, for instance, all thirty-three animals examined on 12 June 1941 gave milk with a lower Δ in the morning, whilst, on 17 December 1941, twenty-nine out of thirty-eight cows gave milk with a lower Δ in the evening. The regularity was not always as pronounced as in the first example, but it was sufficiently marked to warrant the conclusion that cows living under the same conditions of climate and management show evidence of a common factor controlling the osmotic condition of their milk. Its nature is, as yet, unknown.

Another trend is revealed by examining the differences between morning and evening milk over longer intervals. Fig. 1 shows that from May to December 1941 the average morning values were persistently lower than the corresponding evening values. From December 1941 to April 1942 the differences were, on the whole, much smaller, and the Δ values for morning milk tended to be higher than those for evening milk. From the end of April 1942 the morning values again became persistently lower than the corresponding evening values. The periods from April to 1 December 1941, and from 20 April 1942 onwards, coincided with the time during which the cows were kept out of doors day and night, while the period from 1 December 1941 to 20 April 1942 coincided with the time during which the cows were housed at night.

The relationship between the Δ values of morning and evening milk is thus linked with the changes in the temperature of environment to which the cows are exposed. During the major part of the year the animals were kept out of doors day and night, and were thus exposed to colder temperature during the day. During this period the Δ of the morning milk was found to be lower than that of the corresponding evening milk. During the winter the cows were housed at night, and thus exposed to warmer temperatures than during the day, and this reversal leads to a reversal in the Δ relationship, the morning samples now tending to higher freezing-point depressions than evening milk. At all times, therefore, the higher Δ value was obtained at the milking following that period of the day during which the surrounding temperature was warmest.

On the basis of these findings it is possible to explain the apparently contradictory observations on the relationship between morning and evening milk discussed in the introduction. Whether or not a difference in Δ between morning and evening milk is found, and whether this difference is of positive or negative sign, will depend on the time of year chosen for an experiment, and on the conditions under which the cows are living.

Thus, under conditions similar to those obtaining in the present work, data collected during the winter should tend to give higher Δ values for morning milk, whilst data collected during the warmer seasons should lead to the opposite result. The investigations of Aschaffenburg & Temple(7) and of Monier-Williams(1) confirm this conclusion. Aschaffenburg & Temple tested three cows (belonging to the same herd as that used in the present work) frequently from March to October 1936. For each cow the average Δ value for morning milk was found to be 0·002° C. lower than that for evening milk. This result confirms our findings for the corresponding period in 1941. Monier-Williams's data were collected in an area in which conditions were similar to those of the present investigation. His assertion that morning milk has a higher Δ than evening milk appears to arise from the fact that most of his samples were collected during the early months of the year. It cannot, of course, be postulated that the relationship between the Δ of morning and evening milk will be the same for other countries with markedly different conditions of climate and management.

Figs. 2 and 3 show that for a given animal the magnitude of the difference in Δ between morning and evening milk varies in an irregular manner. The greatest variation in Δ between consecutive morning and evening milk for any one animal did not, as a rule, exceed 0·020° C. The largest difference observed during this investigation was one of 0·029° C. (Δ a.m. = 0·545° C.; Δ p.m. = 0·574° C.), the next largest being 0·027° C. (Δ a.m. = 0·543° C.; Δ p.m. = 0·570° C.) and 0·023° C. (Δ a.m. = 0·536° C.; Δ p.m. = 0·559° C.). In

most cases, the largest variations occurred during May or June with a higher Δ value for the evening milk.

C. *The effect of breed*

As the Institute herd contains some Guernsey cows in addition to Shorthorns, the opportunity was taken to compare the Δ values of two breeds of cattle kept under identical conditions. Four animals of each breed were sampled, and the results of 364 tests on Guernsey milk compared with a similar number of results from Shorthorn cows. Samples were taken at frequent intervals from 10 March to 15 July 1942. The average Δ values for each breed and date of sampling are plotted in Fig. 4. There was a

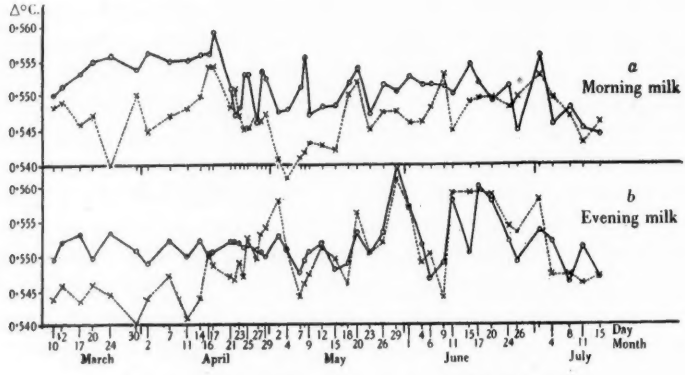

Fig. 4. ○——○ Guernseys. × - - - × Shorthorns.

Table 1

		Average freezing-point depression	
		a.m.	p.m.
(*a*) 10. iii. 42–15. vii. 42			
Guernsey		0·551	0·552
Shorthorn		0·547	0·550
	Difference	0·004	0·002
(*b*) 10. iii. 42–20. iv. 42			
Guernsey		0·555	0·551
Shorthorn		0·548	0·545
	Difference	0·007	0·006
(*c*) 21. iv. 42–15. vii. 42			
Guernsey		0·550	0·551
Shorthorn		0·547	0·550
	Difference	0·003	0·001

marked difference in Δ between the two breeds for the period up to 20 April 1942 during which the cows were housed at night. Afterwards there was little evidence of a breed influence with evening milk, but with morning milk a tendency to higher Δ values for Guernsey milk remained evident. Table 1 contains the average Δ values for the complete experiment (*a*), and for the periods up to (*b*) and after (*c*) 20 April 1942. Apart from the difference of 0·001° C. for evening milk obtained after 20 April 1942, these differences are statistically significant. The magnitude of the differences varies with season, and as the experiment was restricted to observations on only four cows of each breed tested

over part of the year only, it is not possible to draw any definite conclusions on the effect of breed on Δ. The experiment suggests, however, that the breed of cattle is not entirely without effect on the freezing-point level.

D. *The effect of age and state of lactation*

The data of the main experiment did not show any relationship between Δ and the age of the cow or between Δ and its state of lactation.

E. *Correlation between Δ and milk yield*

Correlation tables for 290 samples of morning milk and 285 samples of evening milk gave no evidence of a significant correlation between these two factors.

F. *Correlation between Δ and solids-not-fat content of milk*

As many of our fortnightly tests of the Shorthorn cows coincided with the routine monthly test for fat (Gerber) and solids-not-fat content (by evaporation) carried out by the Chemistry Department, the opportunity was taken to correlate Δ with the solids-not-fat data. The following correlation coefficients were calculated:

	No. of samples	r	t	P
Morning milk	593	+0·12	2·68	<0·01
Evening milk	593	+0·19	4·81	<0·01

indicating a low positive correlation.

The relative constancy of the freezing-point is emphasized by the coefficients of variation for the Δ values and solids-not-fat data:

	Freezing-point depression			Solids-not-fat		
	Mean °C.	S.D.	C. of V.	Mean %	S.D.	C. of V.
Morning milk	0·545	0·0065	1·19	8·86	0·391	4·41
Evening milk	0·548	0·0070	1·28	8·84	0·439	4·97

As the solids-not-fat level, and to some slight extent the Δ level, of milk vary with the breed of cow, misleading results in correlating the two factors may be obtained if the breed of cow is neglected. This is illustrated by the following example:

The data for the milk of three Guernsey and three Shorthorn cows (211 pairs of morning and evening milk) were analysed, the solids-not-fat data being first converted to a fat-free basis. These data yielded a correlation coefficient of +0·42, with $t=6·71$, and $P=<0·01$. Thus, by analysing a mixed population of two breeds, a far higher positive correlation appeared to exist than was found for cows of a single breed.

THE FREQUENCY DISTRIBUTION OF THE FREEZING-POINT AND ITS BEARING ON THE DETECTION OF ADDED WATER IN MILK

The detection of extraneous water in milk requires a close knowledge of the natural distribution of the Δ values of genuine milk, as such knowledge must form the basis for selecting the limiting Δ value for differentiating genuine from adulterated milk.

The Δ distribution for the 1087 morning and 1051 evening samples obtained during the fortnightly testing described above is shown in Fig. 5. The distribution is not normal though it is almost symmetrical in shape; the normal curve proves to be a poor fit

($\chi^2 = 97{\cdot}66$; $P = < 0{\cdot}01$). This result must be expected, since the Δ values are not entirely independent of seasonal and other factors.

The influence of these factors is emphasized when distribution curves for data collected over a limited period are made. This is illustrated in Fig. 6 *a–c*. Fig. 6*a* shows the distribution of all samples tested from May to June 1941. The position and extent of the deviation of the mode from the mean—0·006° C.—indicates a marked positive skewness. Fig. 6*b* gives the distribution of all Δ values determined from June to August 1941, and the reversal of the position of mode and mean, with a deviation of 0·004° C., indicates negative skewness. These examples show that the type of distribution will vary with the design of an experiment, and depend on the period of year during which samples are analysed. If, for instance, values were collected during the winter when, as shown above, the Δ values vary but little, it is likely that a much narrower, symmetrical distribution would be attained, which would rise abruptly to a sharp peak (leptokurtic type

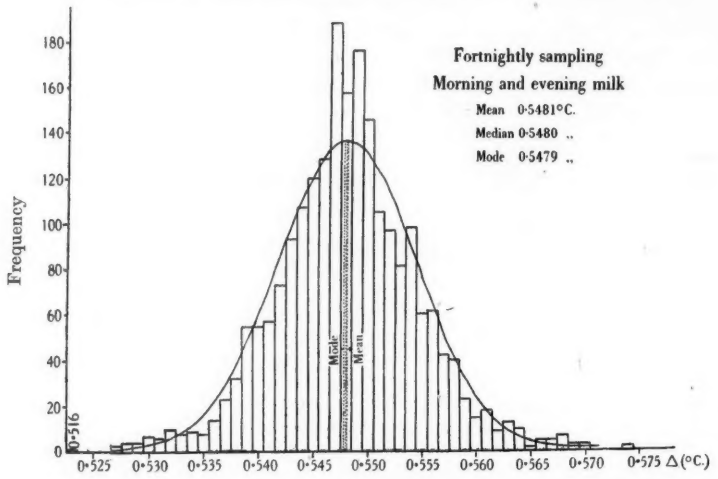

Fig. 5.

of curve). Such a type of curve was indeed found when plotting the data for the winter 1941–2 (Fig. 6*c*).

Only one systematic investigation appears to have been carried out on the shape of the Δ distribution of genuine milk. Tocher (15) has analysed his own data as well as those of Monier-Williams (1) and Bailey (16). For each set of data the curve giving the best fit was of a different type. It is now easy to see why such a result should have been obtained. From our data it follows that no advantage is to be gained by attempting to fit a particular type of curve to a Δ distribution, since the type of distribution will vary with the design of an experiment.

The variations due to factors influencing the Δ distribution must be considered in fixing the limiting value to be used in the differentiation of genuine and watered milk. The current practice in this country is to regard any sample of milk with a Δ of less than 0·530° C. as adulterated, whilst in the United States a more rigid standard is employed by following Hortvet's (2) proposal to allow a tolerance of 3 on the percentage of added water calculated on the basis of an average Δ of 0·550° C. This means that

any sample giving a Δ of less than 0·5335° C. is regarded as watered. The question arises whether these standards, or one of them, should in the light of the results of the present investigation be maintained or modified.

Fig. 5 and, in particular, Fig. 6*a* demonstrate clearly that the American procedure is too stringent, at least under the conditions prevailing in this country: there are too many cases of genuine milk with a Δ equal to, or less than, 0·533° C. for this standard to be applied without risk of misjudgement, especially at the time of year at which the 'spring effect' influences the freezing-point level. This risk is markedly reduced by the relatively small change from a limiting value of 0·5335 to 0·530° C. used in this country. This value makes it highly unlikely that a genuine sample will be falsely judged to be

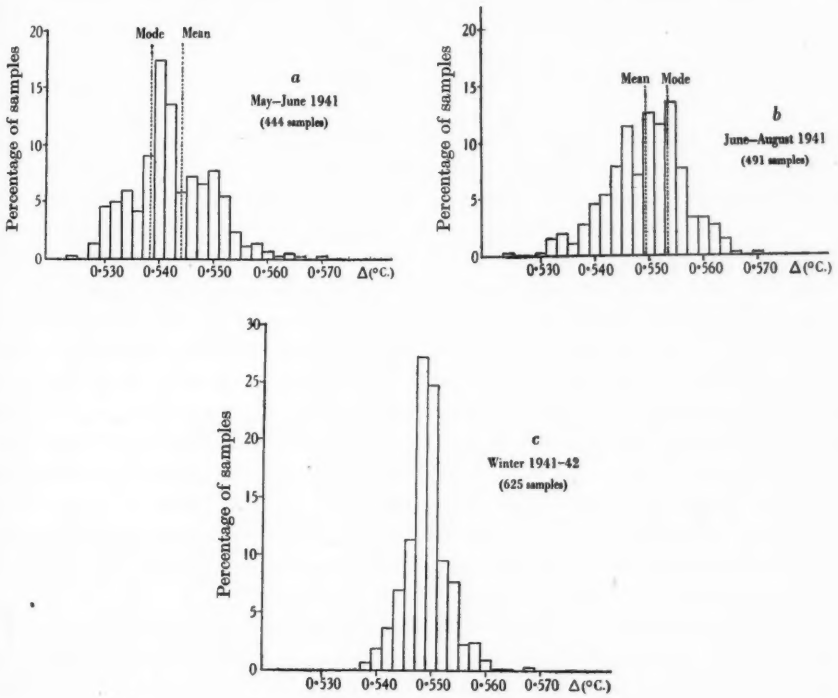

Fig. 6 *a–c*.

adulterated. As shown in Fig. 5 only very few out of more than 2000 tests on the milk of individual cows gave a Δ of less than 0·530° C., and the occurrence of these low values was restricted to a few weeks during the peak of the 'spring effect' in 1941. To reduce the limiting value to a figure of less than 0·530° C., would also reduce the sensitivity of the Δ test and would be too tolerant during the major part of the year (or even all the year round, if, as in 1942, no 'spring effect' occurs, probably owing to the lack of lush spring pasture).

Moreover, in practice most samples submitted for the freezing-point test consist of the mixed milk of a number of animals, and not of the milk of a single cow as in the above considerations. However, the effect of bulking the milk of a number of cows should not

be over-estimated. It has been pointed out that the individual cows of a herd show a uniform tendency towards either high or low Δ values with the result that the Δ value of the mixed milk of these cows may, at certain times, be well above or—what is important—below the general mean. The data for May 1941 in Fig. 2 illustrate the latter effect in producing some remarkably low mean Δ values for the milk of the six cows in question. The following values, for example, were obtained for the morning milk on 21 May 1941:

Cow	Yield (lb.)	Δ° C.
Fancy	18½	0·527
Flora 50	13½	0·534
Flora 45	25¾	0·524
Flora 41	18	0·529
Lottie 15	15½	0·527
Peach 19	9	0·534
	Arithmetic mean	0·5292
	Weighted mean	0·5282

Values as low, or almost as low, as those for the milk of individual cows may thus be expected to occur occasionally with the mixed milk of a number of cows, especially when the mixture comprises morning milk only. Even with the mixed milk of a large number of animals the effect of bulking is not as pronounced as it would be in the case of a normal distribution. This is illustrated by Fig. 1*b* with its three consecutive Δ values of less than 0·540° C. for the mean of the morning milk of over thirty animals.

Turning now to the second basic value used in applying the Δ test, i.e. the reference figure used in estimating the amount of extraneous water believed to be present in an adulterated sample of milk, difficulties similar to those discussed above arise. The current practice most generally followed is that of Hortvet(2) by which the percentage of added water is estimated on the assumption that the Δ of the unadulterated sample is 0·550° C. An estimate on this basis may be far from accurate, since many samples of genuine milk will have a Δ value differing from the assumed mean value of 0·550° C. by an amount equivalent to several units per cent of added water. (This is, perhaps, not always sufficiently realized, since decimal places are not infrequently quoted in stating the amount of extraneous water believed to be present in a milk sample.)

A general mean of 0·548° C. was found in the numerous experiments covering 16 months described in the first part of this paper, but Figs. 1 and 6 show that the average Δ values obtaining at various times of the year vary appreciably. Application of the figure of 0·550° C. during the spring of 1941 would, for instance, in many cases have exaggerated the amounts of added water believed to be present in milk samples tested during that period. It would, therefore, appear wiser, if less impressive, to base the estimate of the amount of added water on the same figure as that chosen for the limiting standard, i.e. 0·530° C., and to state that a sample of milk contains 'a *minimum* of x% of added water' rather than to quote the percentage estimated from an assumed mean value of 0·550° C.

It has not, of course, been the intention of the preceding discussion to give the impression that there is great variability in the Δ values of genuine milk. The range and standard deviations shown in Table 2 emphasize the relative constancy of the freezing-point of milk, a value which is, from the findings of all competent observers, far more constant than any other known and measurable property of milk.

Table 2 shows the extreme range of variations for the fortnightly and bi-weekly experiments described in the first part of the paper.

The greatest range is that shown by the evening milk samples obtained during the fortnightly sampling. Exclusion of the two extreme values (0·516 and 0·574° C.) encountered in this series reduces the range from 0·058 to 0·042° C., and the percentage deviations now become −4·0 and +3·6%, i.e. of the same order as those for the remainder of the results. Thus, with two exceptions, all the Δ values of the milk of the individual cows tested over long periods deviated by less than 4·5% from the mean values.

Table 2

Milking	No. of samples	Mean Δ °C.	Extreme range °C.	% deviation from mean	S.D.
		(*a*) Fortnightly sampling			
Morning	1087	0·547	0·040	−3·7 +3·7	0·0057
Evening	1051	0·550	0·058	−6·2 +4·4	0·0066
		(*b*) Bi-weekly sampling			
Morning	835	0·547	0·042	−4·2 +3·5	0·0058
Evening	800	0·550	0·045	−4·2 +4·0	0·0068

Table 3. *Standard deviations and coefficients of variation of the Δ of morning and evening milk*

	Morning milk		Evening milk		Difference
					C. of v. − C. of v.
Cow	S.D.	C. of v.	S.D.	C. of v.	(a.m.) (p.m.)
Campion 11	0·0041	0·75	0·0051	0·93	−0·18
Flora 61	0·0047	0·86	0·0055	1·00	−0·14
Peach 17	0·0047	0·99	0·0061	1·11	−0·12
Rosalie 14	0·0039	0·70	0·0043	0·78	−0·08
Dorrit 3	0·0044	0·81	0·0054	0·98	−0·17
Flora 50	0·0052	0·95	0·0054	0·98	−0·03
Fâncy	0·0054	0·99	0·0079	1·43	−0·44
Flora 50	0·0078	1·43	0·0083	1·51	−0·08
Flora 41	0·0061	1·11	0·0069	1·25	−0·14
Lottie 15	0·0061	1·12	0·0077	1·40	−0·28
Peach 19	0·0068	1·24	0·0080	1·45	−0·21
Flora 45	0·0065	1·19	0·0078	1·42	−0·23

Standard deviations are a better measure of dispersion than the range of variation which, as just shown, may be considerably affected by a single low or high value. The standard deviations given in Table 2 support the conclusion that the relative constancy of the freezing-point is of a high order. The standard deviations for evening milk exceed those for morning milk, indicating, as already mentioned, a slightly greater variability of the Δ values of the former. This is even more clearly demonstrated by Table 3, showing the standard deviations and coefficients of variation for the Shorthorn cows selected for the bi-weekly sampling. Table 3 shows that in every case the standard deviations and coefficients of variation for evening milk exceed those for morning milk.

SUMMARY

The results of a series of determinations of the freezing-point depressions of the milk of the individual cows of a Shorthorn herd over a period of 16 months are reported.

The relative constancy of the freezing-point was confirmed, all but two values deviating by less than 4·5% from the mean Δ values.

Significant seasonal trends were observed, especially during the spring when the supply of ample amounts of young grass may cause the production of milk with somewhat smaller freezing-point depressions.

Systematic trends in the differences between the Δ of morning and evening milk were found, the evening values being generally higher, except for the winter period, during which the cows were housed at night, when the morning Δ values became the higher. The differences appear to depend on the changes in the temperature of environment to which the cows are exposed. These observations provide an explanation for the discrepancies reported in the literature.

A small, but significant, difference in the Δ level due to breed was observed in a comparison of the milk of Shorthorn and Guernsey cows.

No evidence was found of any influence of age or state of lactation of a cow on the Δ values.

No relation could be detected between milk yield, and only low positive correlation between the solids-not-fat content of milk, and its Δ.

The effect of the seasonal and other variations on the distribution of Δ values is discussed with particular regard to the problem of detecting the presence of extraneous water in milk. It is shown that the limiting Δ value of 0·530° C. commonly used for the differentiation of genuine milk from adulterated milk is well chosen. In reporting on an adulterated sample it is shown to be advantageous to state the *minimum* percentage of added water calculated on the basis of this value in preference to the current practice of attempting to estimate the probable amount of extraneous water from an assumed mean Δ value.

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309. THE FREEZING-POINT OF MILK

III. A SURVEY OF PRODUCERS' MILK AS DELIVERED TO CREAMERIES IN ENGLAND AND WALES

By R. ASCHAFFENBURG, P. L. TEMPLE and B. C. VEINOGLOU
National Institute for Research in Dairying, University of Reading

(With 3 Figures)

Accidental or deliberate addition of water to milk—best revealed by applying the freezing-point test—has been stated to be a common occurrence. No exact information is, however, available about the general extent and frequency of adulteration, presumably because the customary procedures for determining the freezing-point are slow and laborious, and do not lend themselves to the collection of data sufficient in number to warrant statistical treatment and deductions. The development, by Temple(1), of an apparatus which, whilst giving results identical with those obtained in the Hortvet apparatus, greatly increases the speed and convenience with which data may be collected, has made it possible to study the extent of adulteration in a number of districts in England and Wales. For this purpose more than 4000 samples, each representative of the milk of a single producer, were taken on their arrival at various creameries. Although the picture obtained by analysing samples at this stage is incomplete, because further water will, no doubt, find its way into milk on occasion before it reaches the consumer, the survey was deliberately restricted to this stage for the reasons that (1) it is difficult to detect the presence of small amounts of extraneous water after milk has been bulked in large quantities; (2) the freezing-point test should be applied at a time at which the acidity of the milk does not differ much from that obtaining when the milk leaves the udder. Unless this is done, rather arbitrary corrections have to be applied which would inevitably lead to some distortion of the picture.

Experimental

The work was carried out in three parts:

(1) The main survey from March 1938 to July 1939 in which fourteen creameries in England and Wales were visited and a total of 2167 samples was tested. Each of these samples, taken at random, contained the mixed milk from two consecutive milkings of the cows of an individual producer.

(2) A second, smaller, survey during December 1940, made to obtain an indication of the effect of war-time conditions. 454 samples were collected at four creameries previously visited during the main survey. These samples were brought to the National Institute for analysis. Their make-up was the same as that of the samples in the main survey.

(3) A third survey carried out between January and June 1942. In this survey samples representative of the milk produced in two counties of England (designated hereafter as X and Y) were examined. The survey was made in two sections. 658 samples from different producers in county X and 447 samples from different producers in

county Y were tested during January to early April 1942, i.e. under conditions of winter feeding. 218 samples from county X and 240 samples from county Y were again analysed during May 1942, i.e. under conditions of spring feeding. By testing 658 samples in county X almost every producer (excepting producer-retailers) in this county was included in the survey. The number of producers in county Y being much larger, only about one out of every ten producers was represented by the testing of 447 samples originating from this county. The samples taken in county X consisted of milk from a single churn of morning milk, whilst those taken in county Y were made up either from one or two churns of either morning or evening milk, or from the mixed milk of two consecutive milkings. All samples were tested at the National Institute. Those from county Y were preserved with 0·01% mercuric chloride to prevent souring during a relatively long transport period. A correction of 0·004° C. was applied to the results of the freezing-point test on the preserved samples.

The freezing-point depression (Δ) of each sample was determined in an improved model of the Temple apparatus (1).

To guard against serious errors introduced by developed acidity, the titratable acidity of each milk sample was measured at the time of testing. An arbitrary limit was fixed—all samples with an acidity, expressed as per cent lactic acid, as great as, or greater than, 0·20% being discarded. The great majority of samples showed an acidity of less than 0·18% at the time of testing.

Results

The results of the surveys are summarized in Figs. 1–3 showing the Δ distributions on a percentage basis. The lowest Δ encountered during the investigations was one of 0·334° C., indicative of the presence of approximately 40% of added water. Of the remaining samples with a Δ of less than 0·500° C., most fell within the range 0·450–0·499° C.

In a previous communication (2) it was shown that the distribution of the Δ values of genuine milk varies under the influence of seasonal and other factors. There is, thus, no 'standard distribution' with which to compare the results obtained in the present investigation, and this makes it difficult to arrive at an accurate estimate of the frequency of adulteration. Samples with a Δ of less than 0·530° C. are, no doubt, almost without exception adulterated, but it is impossible to assess with any degree of certainty the proportion of samples with a Δ equal to, or slightly exceeding, 0·530° C. which may contain a little added water. The evaluation of the frequency of adulteration given below has, therefore, been based solely on the number of samples with a Δ of less than 0·530° C., and it should be borne in mind that the result thus arrived at gives an under-estimate of the real situation.

Owing to the natural Δ variations of genuine milk and the fluctuations in its mean Δ value (see ref. (2)), the percentage of added water in an adulterated sample of milk cannot be stated with a high degree of accuracy. The results obtained in the present work have, therefore, been grouped, and the percentage of added water in samples with a Δ of less than 0·530° C. estimated on the following basis:

Δ (° C.)	Assumed percentage of added water
0·520–0·529	4
0·510–0·519	6
0·500–0·509	8
<0·500	10

The degree of adulteration has then been assessed by calculation of the percentage of added water present in the bulk of the milk examined, making the further assumption that an equal quantity of milk was delivered by each producer.

The results of this analysis are listed in Table 1 for each creamery or, in the 1942 survey, for each county and period of testing separately.

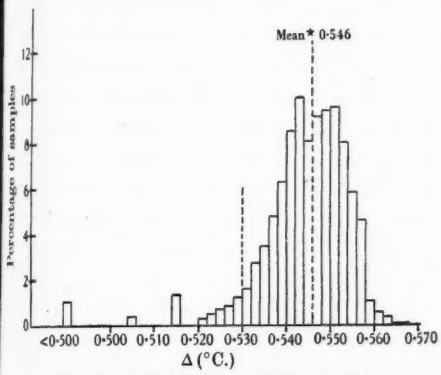

Fig. 1. 1938–39 survey (2167 samples).

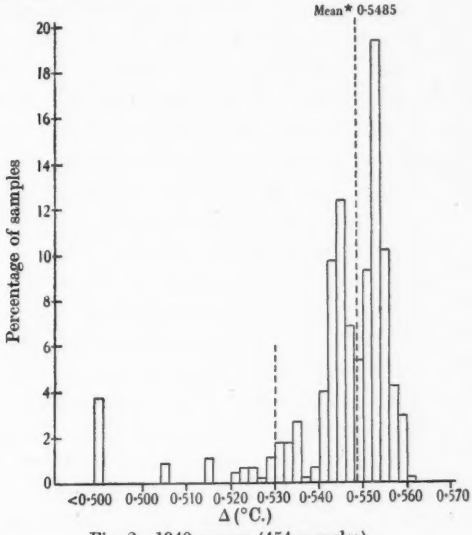

Fig. 2. 1940 survey (454 samples).

* Excluding samples with $\Delta < 0{\cdot}530°$ C.

Table 1

Creamery	Month and year of sampling	No. of samples tested	No. of samples with Δ $<0{\cdot}530°$C.	Percentage of samples with Δ $<0{\cdot}530°$C.	Percentage of added water
1938–9 Survey					
A	Apr. 1938	200	7	3·5	0·18
B	June 1939	64	0	0	0
C	Dec. 1938	267	16	6·0	0·34
D	Apr. 1938	194	17	8·8	0·37
E	May 1939	20	3	15·0	0·90
F	Apr. 1938	71	3	4·2	0·25
G	Aug. 1938	63	11	17·5	0·96
H	Mar. 1938	122	3	2·5	0·16
I	July 1938	27	6	22·2	1·66
J	Feb.–May 1939	442	42	9·6	0·54
K	July 1939	224	4	1·8	0·09
L	June 1938	150	11	7·3	0·47
M	July 1939	163	4	2·5	0·14
N	Apr. 1938	160	10	6·3	0·36
	Total	2167	137	6·3	0·49
1940 Survey					
A	Dec. 1940	99	9	9·1	0·53
D	Dec. 1940	92	3	3·3	0·09
G	Dec. 1940	181	18	10·0	0·85
L	Dec. 1940	82	10	12·2	0·71
	Total	454	40	8·8	0·64
1942 Survey					
County X	Jan.–Apr. 1942	658	50	7·6	0·49
County X	May 1942	218	14	6·4	0·43
County Y	Mar.–Apr. 1942	447	23	5·1	0·32
County Y	May 1942	240	10	2·4	0·25
	Total	1563	97	6·2	0·40

Discussion of results

The data presented in Table 1 and Figs. 1–3 show that, on the average, more than six out of every 100 samples examined gave Δ values indicative of the presence of extraneous water which, either by accident or design, had found its way into the milk. Fortunately, cases of severe adulteration were comparatively rare—in the main survey only 23 out of 2167 samples were found to contain 10% or more of extraneous water—so that the milk, after it had been bulked at the creameries, contained, as a rule, less than 1% of added water (Table 1, last column). Nevertheless, the effect of this degree of adulteration is far from negligible. If we accept the data of the main survey as representative of the general conditions in England and Wales with an annual output of some 1300 million gallons of milk(3), it follows that about 6·5 million gallons of water find their way into milk per annum and are paid for at the price of this commodity. Considering the nutritional aspect, the loss of food value of generally less than one hundredth of the solid matter contained in a unit volume of milk cannot be regarded as serious when compared with other actual or potential sources of damage to the nutritive value of milk.

The detailed data given in Table 1 show that the frequency and degree of adulteration vary considerably from creamery to creamery. The impression was gained during the investigations that these variations are as much a reflection on the efficiency of control and supervision of the incoming milk supplies exercised by the creameries as on the efficiency or integrity of the producers in the various areas. The data for creameries B, H, K and M in the 1938–9 survey show that it is possible to attain a very high standard of efficiency. This observation encourages the belief that a similar standard could—and should—be reached by all milk collecting centres. This task should be greatly facilitated by the wider adoption of the freezing-point test as a routine method of control.

The results obtained in the small, 1940 survey (Fig. 2) indicated a slight deterioration in the position under war-time conditions, but a comparison of the data given in Table 1 with those for the 1938–9 survey shows that there was no uniform trend to increased adulteration, the values for two of the creameries (D and G) being, in fact, smaller than those obtained at the same creameries prior to the war. The 1942 survey, carried out on a more adequate scale, shows that the position had remained virtually unchanged after $2\frac{1}{2}$ years of war—a gratifying result considering the increased temptation to water milk brought about by the increased demand for this commodity and the higher price level.

The 1942 survey was, as mentioned before, arranged in two parts. Fig. 3 *a*, *c* summarize the results obtained whilst herds were on winter feed, Fig. 3 *b*, *d* those obtained after the animals had been turned out to grass in the spring. The mean Δ values given in Fig. 3 were calculated after exclusion of the samples with a Δ of less than 0·530° C. which are undoubtedly adulterated. It is realized that not all the remaining samples are necessarily genuine, but it is unlikely that the proportion of samples containing a few units per cent of added water is such as to change the distributions much from those which would have been obtained if all the samples were strictly genuine. It will be seen that for both counties the Δ level found under conditions of spring feeding was slightly lower than that obtaining during the winter period. This is in accordance with the observation of Aschaffenburg & Veinoglou(2) that there is a tendency to lower Δ values during the spring. As it happened, in the spring of 1942 the tendency was not pronounced owing to the lack of lush spring pasture, but, nevertheless, it makes itself

felt in the results shown in Fig. 3. For the milk from county Y tested during May 1942 a mean Δ as low as 0·542° C. was found, and very few Δ values greater than 0·550° C. were encountered during this period. These observations serve to substantiate the conclusion reached in other work (2) that, in view of seasonal variations, it would be unwise to calculate the percentage of added water in an adulterated sample of milk from an assumed mean Δ value for genuine milk of 0·550° C.

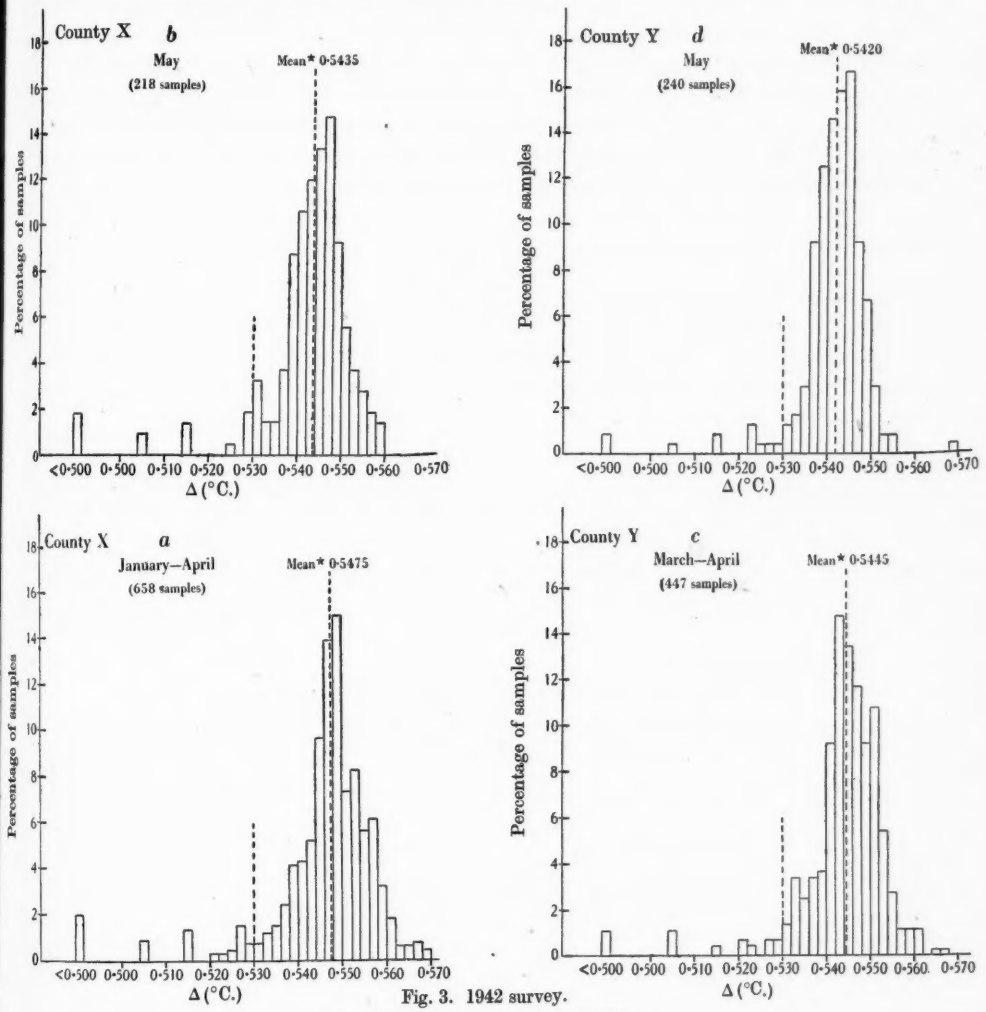

Fig. 3. 1942 survey.

* Excluding samples with Δ < 0·530° C.

All samples included in the 1942 survey were also analysed for their solids-not-fat content. The solids-not-fat distributions for the milks from the two counties obtained during the winter period were found to differ markedly. The milks from county X had a satisfactory solids-not-fat level, the average, after exclusion of all samples with a Δ of less than 0·530° C., amounting to 8·74 %, but many of the samples obtained in county Y

were poor in non-fatty solids, the corresponding average amounting to only 8·51%. (For details see S. J. Rowland(4).) Thus, nearly 50% of these samples failed to reach the presumptive standard of 8·50%. No less than sixty-seven out of 447 samples had a solids-not-fat content of less than 8·20%, of which only thirteen were, according to the freezing-point test, adulterated. These data show that *a low solids-not-fat content is not a safe criterion for the genuineness or non-genuineness of a milk sample*, and emphasize the necessity for confirming the suspected presence of added water by means of the freezing-point test. During the second period of sampling, i.e. after the herds had been turned out to grass, the solids-not-fat level of the samples from county Y showed a material improvement, the average increasing to 8·68%, whilst no change was observed for the milk from county X. Fig. 3 *c*, *d* illustrates the lack of relationship between solids-not-fat content and Δ, the data in Fig. 3*d* showing a decrease in the Δ level at a time at which the solids-not-fat level had increased considerably.

SUMMARY

The results of freezing-point tests on some 4000 samples of milk as delivered by individual producers to creameries in England and Wales are analysed. Prior to the war about six out of every 100 samples were found to contain added water, but, as cases of heavy adulteration were comparatively rare, the total effect on the bulked milk supplies was small. After 2½ years of war the position had remained virtually unchanged.

The authors wish to thank Prof. H. D. Kay, Dr S. J. Rowland and Dr P. White (Department of Physics, Reading University) for much valuable advice and criticism. They are grateful to all those who helped to arrange the collection of samples, especially to Dr A. L. Provan (Harper Adams Agricultural College, Newport) and Mr J. McClemont.

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637.127:576.8:637.323

310. THE INFLUENCE OF 'ABNORMAL' MILK UPON THE YIELD AND QUALITY OF CHEDDAR CHEESE*

By C. K. JOHNS† and C. A. GIBSON,‡ *Department of Agriculture, Ottawa, Canada*

In studies previously reported (1) it was found that milk of normal appearance from cows having a latent infection with *Streptococcus agalactiae* yielded less cheese than that from cows with normal udders. Milk from cows free from *Str. agalactiae* infection, yet classed as 'abnormal' on the basis of biochemical tests, behaved similarly. The lower yield appeared to result from the lower casein and solids-not-fat contents of these 'mastitis' milks. With a single exception, the quality of the cheese made from these milks was not inferior to that of cheese made from normal milks. These findings appeared to warrant the extension of the studies to ordinary cheese-factory milks.

The previous studies were conducted with the milk of cows in the Central Experimental Farm herd, whose udder history was known from routine bacteriological and other examinations. In dealing with cheese-factory milk supplies, such information was not available. On the basis of previous studies (1) it was decided to use the catalase test, supplemented by a direct microscopic count of leucocytes, as the criterion of abnormality in the selection of herd milks for the present work. The catalase test, using the Davis & McClemont (2) modification, is particularly suitable as a rapid factory test, and has been recommended by Davis (3) as the best single test for this purpose. While this test is not specific for mastitis, there is reason to believe that mastitis is the commonest cause of abnormal milk (4). The demonstration of the presence of *Str. agalactiae* in a number of vats of abnormal milk selected on this basis tends to confirm this view.

Experimental

In Series I the usual factory practice in the making of Canadian Cheddar cheese was followed. In Series II and III, the same practice was followed as closely as possible, small experimental vats of normal and of abnormal milk being so handled as to obtain the greatest possible uniformity in the final product.

Chemical analyses were conducted on the milk, whey and cheese by the Division of Chemistry, Science Service. With the exception of the substitution of the Davis & McClemont modification for that of Hastings (5) in the catalase test, the analytical methods employed were as described in the previous paper (1). The theoretical yield of cheese was again determined by the formula (6)

$$\text{Yield} = (1{\cdot}4\ \text{fat} - 0{\cdot}9)\ N,$$

where N varies with moisture content of cheese from 1·53 for 33% to 1·68 for 39%.

* Contribution from the Division of Bacteriology and Dairy Research, Science Service (No. 168 Journal Series), and the Animal Husbandry Division, Experimental Farms Service, Department of Agriculture, Ottawa.

† Associate Bacteriologist, Division of Bacteriology and Dairy Research.

‡ Creamery Manager, Animal Husbandry Division.

Series I, 14–17 May 1940

An invitation was extended by the manager of a new six-vat factory to conduct a series of experiments there. Unfortunately, the segregation of herd milks as planned could not be carried out without disrupting plant operations. The best that could be done was to segregate the milk of ten suppliers, not all of which was particularly high in catalase content, from the last truck-load received and to make this up separately on two successive days. On the following 2 days attempts to make up smaller lots of normal milk as controls in a small experimental vat resulted in spoilt vats. To afford some basis for comparison, records were obtained on four vats made from the ordinary run of milk during the same 2 days on which the abnormal vats were made. (One vat of washed curd cheese was not included.)

Table 1. *Data from normal and abnormal milks*, 14–17 *May* 1940

Type of milk ...	Normal		Abnormal	
Vat no.	N1	N2	382	386
		Milk		
Catalase %	18	19	32	26
Fat %	3·1	3·2	3·1	3·0
S.N.F. %	8·48	8·55	8·31	8·09
Casein %	2·14	2·18	1·92	1·95
Casein no.	79	80	75	76
Chlorides %	0·100	0·100	0·109	0·111
Globulin N %	0·0311	0·0318	0·0378	0·0390
		Whey		
Fat %	0·27	0·24	0·36	0·34
S.N.F. %	6·49	6·42	8·98	9·02

Table 2. *Data from vats made from abnormal and average milks*, 14–15 *May* 1940

Type of milk ...	Average				Abnormal	
Vat no.	380	381	384	385	382	386
			Milk			
Fat %	3·0	3·1	3·2	3·1	3·0	3·1
			Cheese			
Moisture %	34·52	33·86	33·65	33·77	36·18	35·38
Fat %	34·94	35·44	35·45	34·13	33·51	32·53
Total solids %	65·48	66·14	66·35	66·23	63·82	64·62
Yield per 100 lb. milk	8·25	8·07	8·05	8·15	7·96	7·59
Theoretical yield	8·0	8·05	7·90	8·31	8·44	8·10
Actual/theoretical %	103·1	100·3	101·9	98·1	94·3	94·7
Average %		100·85			94·0	

Influence upon yield of cheese

Analytical data from the normal and abnormal milks (Table 1) show definitely lower values for the latter in solids-not-fat, casein, and casein number, and higher values for catalase, chlorides and globulin N. Even more marked differences are evident in the values for the whey samples. Judged by results previously reported (1), these differences in composition of the wheys are of greater magnitude than would be expected on the basis of catalase values.

When the vats made from the abnormal milk are compared with those made from the ordinary run of milk (Table 2), it is evident that the lowered contents of casein and

solids-not-fat of the abnormal milks indicated in Table 1 are reflected in a lower yield of cheese, i.e. only 93·2% of that for the unselected milks used as controls.

Influence upon quality of cheese

The flavour scores of the abnormal and control vats are shown in Table 3. One of the abnormal vats (no. 386) was placed in second grade on the first and second gradings, but improved enough to obtain the minimum score for first grade on the final grading. The other abnormal vat compared favourably with the four control vats. No significant differences were noted in body and texture.

Table 3. *Flavour scores of cheese made* 14–15 *May* 1940

Type of milk ...	Control				Abnormal	
Vat no.	380	381	384	385	382	386
Date of scoring						
1 June 1940	39·5	40·0	39·5	40·0	39·5	38·0
8 June 1940	39·2	39·8	39·0	39·0	40·0	38·0
5 Oct. 1940	39·5	39·0	39·5	39·0	39·5	39·0

N.B. To be classed as first grade under the Dominion grading regulations, cheese must have a flavour score of not less than 39 points and a total score of not less than 92 points.

SERIES II, 18–20 JUNE 1940

In order to avoid the difficulties encountered in segregating and manufacturing cheese from normal and abnormal milks in a commercial factory, 400–500 lb. lots of each type of milk were selected daily for 3 days at a nearby factory and transported to the Central Experimental Farm Dairy for manufacturing under conditions affording closer control. In selecting the herd milks for the two groups, the catalase test was supplemented by the leucocyte count (7).

Influence upon yield of cheese

A portion of the data obtained is shown in Table 4. It will be seen that the various tests employed to indicate abnormalities in the milk often failed to agree. For example, the leucocyte counts showed wide differences between the two classes of milks, while this was not reflected to the same degree in the catalase values. Subsequent studies indicate that some of the catalase readings may have been unduly high, due to the use of cork stoppers fitted with capillary tubing in place of pierced rubber stoppers as specified by Davis & McClemont. Furthermore, the solids-not-fat were proportionately higher in the abnormal milks, while the same was true of the casein content. Only slight differences were found in the casein numbers and globulin contents, while the chloride contents of the abnormal milks were slightly higher. Values for initial *p*H and titratable acidity also showed some differences.

If the degree of abnormality is judged by the cell count, initial acidity, *p*H, or chloride content, one would expect a somewhat lower yield from the abnormal vats. If, on the other hand, the solids-not-fat or casein values are taken as criteria, a yield at least equal to that from the normal milk would be expected. As the data show, on the basis of the theoretical yield the normal vats yielded slightly less cheese in each instance. This correlates with the higher losses in the whey from the normal vats.

At the suggestion of Dr W. V. Price (8) the yield was also calculated on the basis of the percentage of milk solids recovered in the cheese. On this basis the yield from the normal vats was only 97·3% of that from the abnormal.

Table 4. *Data from Series II*, 18–20 *June* 1940

Type of milk ...	Normal			Abnormal		
Vat no.	1	3	5	2	4	6
Initial acidity	0·172	0·167	0·165	0·150	0·155	0·160
Initial *p*H	6·50	6·62	6·68	6·77	6·71	6·78
Catalase %	28	13	28	37	36	33
Cell count (thousands)	111	130	130	2040	1550	1570
% starter	0·75	1·0	0·75	0·75	1·0	0·75
Acid at setting %	0·20	0·19	0·195	0·20	0·195	0·195
Minutes to 1st coagulation	6	6	8	—	5·5	6·5
Milk						
Fat %	3·21	3·35	3·25	3·24	3·08	3·23
S.N.F. %	8·22	8·30	8·53	8·34	8·40	8·47
Casein %	2·23	2·18	2·27	2·32	2·29	2·32
Casein no.	77	78	79	77	77	78
Chlorides %	0·101	0·107	0·102	0·110	0·109	0·112
Globulin N %	0·0334	—	0·0295	0·0338	—	0·0349
Whey						
Fat %	0·27	0·29	0·25	0·22	0·23	0·22
S.N.F. %	6·23	6·13	6·29	6·05	6·02	6·13
Cheese						
Moisture %	34·66	34·70	35·09	35·76	36·00	36·67
Fat %	34·53	35·17	37·20	33·59	32·61	32·10
Total solids %	61·68	61·85	61·30	60·71	60·33	59·81
Yield per 100 lb. milk	8·39	8·35	8·64	8·70	8·47	8·85
Theoretical yield	8·50	8·72	8·60	8·70	8·37	8·80
Actual/theoretical %	98·7	95·8	100·3	100·0	101·2	100·3
Average %		98·27			100·5	
% milk solids recovered in cheese	46·8	44·8	47·5	47·9	47·6	47·5
Average %		46·37			47·67	

Influence upon quality of cheese

The flavour scores in Table 5 show no significant differences after 3 weeks' curing. However, subsequent gradings after 3 and 9½ months showed a pronounced drop in score for all three cheese made from abnormal milk. No significant differences in body and texture were found.

Table 5. *Flavour scores on cheese made* 18–20 *June* 1940

Type of milk ...	Normal			Abnormal		
Vat no.... ...	1	3	5	2	4	6
Date of scoring						
6 July 1940	39·0	40·0	39·2	39·5	39·2	39·1
28 Sept. 1940	38·5	39·0	39·3	37·5	37·5	38·0
4 Apr. 1941	39·5	40·0	39·0	37·5	37·5	37·5

Series III. 18–23 October 1940

Since the results from Series II differed from those in previous studies in failing to show a lowering in yield but in showing a deterioration in quality of cheese from abnormal milk, these studies were repeated in October 1940. Unfortunately, bacteriophage infection(9) ruined the results from the first 2 days, all four vats scoring second or third grade for flavour, and also being poor in texture. A starter resistant to this phage was therefore obtained and used to make three more pairs of vats.

Influence upon yield of cheese

As will be seen from the data in Table 6, there was again a lack of agreement between the results of the various tests used to indicate 'abnormality'. Neither the initial acidities, *p*H values, solids-not-fat, nor casein contents indicated significant differences between the two types of milk employed; the chloride contents and casein numbers showed a slightly greater difference, but not nearly as marked as in the catalase values and leucocyte counts. In this series, pierced rubber stoppers were employed for the catalase test and the test run in triplicate. This may explain the better agreement between catalase values and leucocyte counts for the normal vats than was found in Series II.

Table 6. *Data from Series III*, 18–23 *October* 1940

Type of milk ...	Normal			Abnormal		
Vat no.	5	7	9	6	8	10
Initial acidity	0·145	0·15	0·14	0·145	0·15	0·145
Initial *p*H	6·58	6·64	6·70	6·64	6·71	6·71
Catalase %	16	18	17	50	52	46
Cell count (thousands)	370	115	213	1900	1840	1940
% starter	1·25	1·25	1·25	1·25	1·25	1·25
Acid at setting %	0·19	0·19	0·19	0·19	0·19	0·19
Minutes to 1st coagulation	8	9	8	7	9	8
Milk						
Fat %	4·0	3·98	3·88	3·75	4·07	3·93
S.N.F. %	8·4	8·71	8·46	8·33	8·75	8·68
Casein %	2·47	2·38	2·30	2·40	2·52	2·48
Casein no.	81	80	79	78	78	78
Chlorides %	0·126	0·122	0·123	0·132	0·132	0·132
Globulin N %	0·0200	0·0148	0·0198	0·0181	0·0248	0·0206
Whey						
Fat %	0·37	0·42	0·40	0·31	0·36	0·36
S.N.F. %	6·50	6·85	6·77	6·33	6·67	6·67
Cheese						
Moisture %	37·21	35·03	35·88	38·20	36·44	36·68
Fat %	34·55	35·37	33·66	33·34	34·90	33·37
Total solids %	62·79	64·97	64·42	61·70	63·56	63·32
Yield per 100 lb. milk	9·8	9·6	9·12	9·3	9·8	9·7
Theoretical yield	10·42	10·0	9·8	9·9	10·45	10·18
Actual/theoretical %	93·8	96·0	93·1	94·0	93·8	95·3
Average %		94·3			94·4	
% milk solids recovered in cheese	48·45	46·6	47·2	46·8	48·5	48·1
Average %		47·42			47·8	

The data in Table 6 show only slight differences in yield between the two types of milk, whether calculated on the basis of actual/theoretical yield or on percentage of milk solids recovered in the cheese.

Influence upon quality of cheese

As in Series II the flavour scores shown in Table 7 indicate an advantage for the cheese made from normal milk. Vat 8 was the only vat made from abnormal milk in the two series which failed to show a significant deterioration in flavour subsequent to the initial scoring. No explanation can be offered for the development of 'rancid' flavour in vat 6. Despite fairly extensive studies which have been made in these laboratories to determine the cause of this, the most serious flavour defect in Canadian Cheddar cheese,

present knowledge furnishes no clue as to why 'rancid' flavour appeared in only one out of three vats made from the milk of the same herds.

Table 7. *Flavour scores on cheese made* 18–23 *October* 1940

Type of milk ...	Normal			Abnormal		
Vat no. ...	5	7	9	6	8	10
Date of scoring						
19 Nov. 1940	38·5	39·0	39·5	37·0*	38·5	39·0
4 Apr. 1941	39·5	38·3	39·5	36·0†	38·8	37·0

* Slightly rancid. † Rancid.

Discussion

The outstanding feature of these studies appears to be the discrepancy between the results of Series II and III and those of Series I and of the former studies (1). While the yields from the normal and abnormal milks are in agreement with the respective solids-not-fat and casein contents, it is hard to understand why some milks showing high catalase values and leucocyte counts should be more normal in chemical composition than others showing equally high values for catalase and leucocytes. From the standpoint of cheese yield, it would appear that the solids-not-fat and casein contents are the more reliable criteria of normality.

A further difference between the results of Series II and III and those of the preceding studies lies in the flavour deterioration in the cheese made from abnormal milk, subsequent to the initial grading. The explanation for this difference is no more apparent than for the differences in yield. While the moisture contents of the 'abnormal' cheese were generally higher than those of the normal, these differences are less than those found in Series I, where no such deterioration in quality was noted.

In the previous paper (1) it was concluded that cheese yields were lower from 'mastitis' milk. Marquardt & Needham (10) disagree with this conclusion, stating that it cannot be accepted on the evidence presented. The basis for this view is not clear from their remarks, but appears to rest upon the differences in moisture content, and the relatively lower moisture contents of these cheese than of Cheddar cheese manufactured in the U.S.A. This difference in moisture content is the result of the greater emphasis upon firm body in Canadian Cheddar cheese-making. Marquardt & Yale (11) examined thirty-four entries of 'export Cheddar' at the 1941 New York State Fair, and found moistures ranging from 35·0 to 41·5%, with an average of 37·3%. This may be compared with the results reported by Hood & White (12) in 1927 for Canadian Cheddar cheese offered for grading. For 227 samples of first-grade cheese, the moisture content varied between 30·75 and 38·84%, with an average of 34·07%. Samples criticized as being 'slightly weak' in body, however, averaged approximately 2% higher moisture content than those classed as 'perfect' and 'good'. While in our studies the moisture content of the cheese made from abnormal or 'mastitis' milk was generally somewhat higher than that of cheese made from normal milk, it was not so high as to affect the body or texture.

Since the cheese made from abnormal milk usually contained more moisture than those made from normal milk, it may be argued that the calculation of yield by means of a formula which compensates for differences in moisture content is misleading. The same could be said of the calculation of the percentage of milk solids recovered in the

cheese. We have therefore calculated the yields for sixteen vats reported in our previous publication (1), as well as those in the present series, on the basis of the formula (6)

$$\text{Yield} = 2{\cdot}3\ \text{fat} + 1{\cdot}4,$$

and compared the theoretical yield so calculated with the actual yield. The average values for each group of vats are shown in Table 8.

Table 8. *Average theoretical yields of cheese. Calculated on the basis of formula: Yield = 2·3 fat + 1·4*

Date	Type of milk	No. of vats	Average % moisture in cheese	Yield of cheese		
				Actual	Theoretical	Actual / Theoretical %
Jan. 1939	Normal	4	36·94	10·28	9·64	106·64
	Abnormal	3	36·53	9·97	9·91	100·61
	'Mastitis'	1	38·77	9·62	9·25	104·0
Apr. 1939	Normal	4	35·38	9·73	10·17	95·67
	Abnormal	2	36·63	9·02	9·68	93·25
	'Mastitis'	2	34·98	8·64	9·80	88·2
Apr. 1940	Control	4	33·95	8·13	8·47	96·0
	Abnormal	2	35·78	7·78	8·41	92·51
June 1940	Normal	3	34·82	8·46	8·88	95·27
	Abnormal	3	36·14	8·67	8·72	99·4
Oct. 1940	Normal	3	36·04	9·51	10·49	90·6
	Abnormal	3	37·11	9·6	10·39	92·43

While the advantage for the normal vats in the first three series is generally less marked where differences in the moisture content of the cheese are ignored, there is still a difference in their favour. With the June 1940 series, where the abnormal vats averaged 1·32% higher moisture content, the difference in favour of the abnormal vats is greater than when yields are calculated on the basis of the other formulae (Table 4). In the October 1940 series, the results are in closer agreement with those shown in Table 6.

Throughout these studies there has been good agreement between the solids-not-fat content of the milk and the yield of cheese. The comprehensive studies of Rowlands (4) and others have shown that subclinical mastitis accounts for a very high proportion of milks low in solids-not-fat and produces a large decrease in these constituents. It does not seem unreasonable, therefore, to expect a lower yield of cheese from mastitis milk, as reported by Price (13), Mattick *et al.* (14) and Johns, Hicks & Gibson (1). Both Price and Mattick have also reported that cheese made from 'mastitis' milk is inferior in flavour and body to that made from normal milk. Our results in Series II and III indicate that cheese made from milk which, while normal in many respects, is high in catalase and leucocytes may fail to maintain its initial flavour score on further ripening. Thus abnormal milk may adversely affect either yield or quality of cheese, or both.

We are indebted to Mr S. Labarge of the Chateau Cheese Company, Ottawa, for providing the facilities for the factory studies reported in Series I; to Mr H. Levere of Carsonby, Ont., for assistance in obtaining the milk used in Series II and III; to Mr R. K. Howson, B.S.A.,* Division of Bacteriology and Dairy Research, for technical assistance; to Mr T. J. Hicks, Senior Dairy Produce Grader, Dairy Products Division, for scoring the cheese; and to the Division of Chemistry, Science Service, for chemical analyses of the milk, whey and cheese.

* Killed in action, May 1942.

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311. THE INFLUENCE OF BACTERIOPHAGE ON THE CHEESE-MAKING PROCESS

BY G. J. E. HUNTER, *Dairy Research Institute* (*D.S.I.R.*), *Palmerston North, New Zealand*

(With 1 Figure)

The constant use of a relatively small number of selected pure strains of lactic streptococci as cheese starters in New Zealand has enabled strong concentrations of specific or polyvalent races of bacteriophage to become established in the surroundings of commercial factories. In previous papers (1, 2), the special precautions necessary to prevent failures of starter cultures due to the destruction of the streptococci by phage have been discussed. By the appropriate technique it has been found possible to maintain mother cultures of 'single-strain' starters for an indefinite period of time, while the chances of failure in the bulk starter have been considerably reduced. Complete or partial failure of acid production in the cheese vat may result from any or all of the following causes: (*a*) phage contamination in the starter culture, (*b*) phage contamination in the farmer's milk, (*c*) phage contamination from the vats and equipment. Following the success obtained in eliminating failures from the cultures themselves, it became desirable to ascertain the influence of small amounts of contaminant phage upon the development of acid by the starter organisms in the cheese vat and upon the quality of the cheese produced. Complete failure of the organisms in the vat due to gross phage contamination commonly results in the production of poor quality cheese. Using a culture consisting

of a mixture of streptococci, Johns & Katznelson(3) demonstrated stoppage of acid development in cheese vats due to the action of a polyvalent streptococcal phage and showed that experimental infection of a vat of milk resulted in failure at 'cutting' and production of poor quality cheese with a 'fruity' flavour.

Experience has shown that complete failure of 'single-strain' starters in the cheese vat as a result of contamination of the vats and utensils with phage is rare, but in several instances where the starter has been proved free from infection, vats have been observed to exhibit some degree of slowness towards the end of the cheese-making process. When slow acid development occurs early in the process, particularly before the whey is run off, the partial or complete failure is invariably traceable to the effect of contamination of the bulk starter where phage has multiplied to an extent sufficient to cause lysis of the organisms in the next propagation, i.e. in the vat. It is essential that the starter added to the cheese milk be normal and free from infection before the importance of phage present on the surfaces of factory equipment and in the farmers' cans can be assessed. Complete control over cultures has been obtained in several commercial factories and in the Institute factory where the experiments reported in this paper were carried out.

SOURCES OF PHAGE INFECTION

The primary source of infection in factories is the cheese whey(2) which comes into contact with equipment directly, or by splashing, or is distributed through the air by the atomizing action of the separator. When a starter is in use continuously, a constant re-infection of the surroundings occurs, and certain pieces of dairy plant, as for example milk or whey pumps, may harbour and distribute phage. Dried whey particles and dust from grossly contaminated areas about a factory are potential carriers of infection; phages active against particular strains of streptococci can be isolated readily from dust on the ground under whey tanks. The risk of infection from whey-contaminated dust, however, varies considerably with the lay-out of a factory and with weather conditions. The overhead mechanical gear, particularly that to which the agitators and blades used for stirring the milk and curd are attached, probably forms the most immediate source from which phage gains access to the cheese vats. Again phage has been found, on many occasions, in the milk delivered to factories, especially when farmers take home whey in milk cans for pig feeding. Regenerative flash pasteurization of milk (150–160° F., approximately 30 sec.) as practised in New Zealand cheese factories does not completely destroy the lytic agent which is protected to some extent by the milk proteins.

It appears in practice that any one source of infection of the milk rarely supplies sufficient phage to cause complete failure of the organisms in all vats, but a real danger does exist from the aggregate infection received by the milk at various points between the receiving platform and the vat unless precautions are taken to wash equipment thoroughly. The normal daily routine cleaning of dairy plant is generally sufficient to reduce contamination to the low level necessary to avoid trouble during the 6–7 hr. the starter organisms are required to produce acid in the cheese vat. Once phages become widespread in factories the possibility of ensuring complete absence of infection on large metal and wooden surfaces is remote, and hence the milk in the cheese vats regularly contains some phage which is able to multiply on the susceptible organisms to give a relatively high titre in the whey at 'running' (dipping), approximately 3–3½ hr. from the time of adding the starter.

Method for estimating phage strength

The very small plaque size of many of the streptococcal phages makes accurate counts very difficult. Moreover, in dealing with raw materials such as unfiltered whey, results must be read before the growth of contaminants covers the plate. Consequently the method of placing drops of a series of dilutions of the cheese whey, or of phage solutions, on plates was used during the course of this work as a convenient and rapid method for the estimation of phage strength. The dilutions were prepared in sterile water and a standard drop (from a plate loop 2 mm. diam.) of each placed on a well-dried yeast whey agar plate thickly spread with the susceptible organism. Incubation of the plates at 30° C. gave clear-cut results in less than 8 hr. Where phage was present in the original material in strong concentration, the first few dilutions gave clear confluent areas of lysis, and the higher dilutions gave progressively decreasing numbers of isolated plaques. In this test the titre of the phage is reported as the highest dilution showing plaques.

Tests in many factories revealed the fact that even when a vat appeared to behave normally, the whey at 'running' frequently had a phage titre varying from 10^{-1} to 10^{-8}. On keeping the whey overnight, the phage has sometimes been observed to reach titres of 10^{-12}–10^{-15}. The result varied with the strain of *Streptococcus* used as starter.

Effect of phage in small quantities of milk

It was of interest to determine the concentration of phage necessary to cause a slowing down in the rate of acid production under conditions obtaining in the cheese vat. Trials by means of a modified 'vitality' test (4) were carried out in 150 ml. quantities of pasteurized milk containing varying amounts of cheese whey or of purified (5) phage preparations of medium titre such as would be found in whey from most factories. In liquid milk, a trace of phage, e.g. one part of phage preparation in one hundred million parts of milk, causes lysis of the organisms developed from a 1% bacterial inoculum within 7 hr. at 30–37° C. Acid is generally produced by the streptococci at a steady rate until approximately 10–15 min. before lysis when there is a gradual decrease. Complete cessation of acid development occurs very shortly after lysis of the organisms. In renneted milk, with the bulk of the whey drained from the curd at an early stage, the time elapsing before complete cessation of acid development, as judged by acidity titrations on the free whey, is prolonged, and the phage addition which causes lysis in milk in 7 hr. is not sufficient to cause a similar failure in renneted milk within that time.

During the process of cheese manufacture, the starter organisms are required to grow in the milk and curd in the cheese vats at a temperature of 88° F. for some periods and at 98–102° F. for about 2 hr. during the 'cooking' period. Previous work (6) has shown that the rate of phage multiplication in milk depends to some extent on the strain of *Streptococcus* and the type of phage race involved and upon their reactions to changes in temperature conditions. For this reason it was to be expected that with a phage race which was retarded at the temperature employed for 'cooking' the curd, a higher initial concentration would be required to cause failure of acid production than with one which multiplied rapidly at this temperature. The organisms studied were all strains of *Str. cremoris* (Orla Jensen), and the phage preparations were all of the same order of strength, giving titres of 10^{-7}–10^{-8}. The borderline initial concentration to cause failure of acid

production within 6½ hr. for phages not inhibited at 100° F. was found to be in the vicinity of 1 part phage preparation to 1,000,000 parts of milk inoculated with 1% starter, whereas for races which were retarded at 100° F. the initial concentration required was about 1 part in 1000. Where a strain of *Str. cremoris* was susceptible to a series of phage races, the initial concentration also varied from 1:1,000,000 to 1:1000, and in some cases to more than 1:100, corresponding to the reaction of the different races to temperature of incubation.

Most of the pure strains of *Str. cremoris* used as starters in New Zealand are liable to attack by phages which are active at the higher temperatures, and hence the lower concentration limit is the one most likely to be of importance in considering partial failures in cheese-making. Experiments in small and large quantities of milk are not strictly comparable, but calculation on the above basis suggests that about 1 pint of whey containing such amounts of phage as are frequently encountered in commercial factories would exert a noticeable effect in an 800 gal. vat of milk inoculated with 1% of a 'single-strain' starter. In practice the effect of a given phage infection depends to some extent on the percentage of starter used and the method of cheese manufacture adopted. A higher percentage of starter, although showing the same graded acid production with graded addition of phage, permits a more rapid growth of the organisms and higher acidity figures to be obtained at the end of the test. Thus a more massive inoculation of the cheese milk, e.g. 2% starter instead of the usual 1–1¼%, may serve to preserve the starter organisms until the acidity required in the cheese-making process is attained. In practice, however, an increase in the amount of starter is possible only up to a certain limit because the various cheese-making operations cannot be hastened beyond a certain extent.

Phage added at 'renneting' and also as late as 'cutting' has some effect on subsequent acid development, but here again slightly higher acidities are obtained before a marked slowing down is observed. It is evident, however, that phage is able to diffuse into the curd during the period between 'cutting' and 'running'.

ADDITION OF PHAGE TO THE CHEESE VAT

For the purpose of these experiments a stock solution of hp phage prepared in skim milk and showing a titre of 10^{-12} was employed. Preliminary trials indicated that initial additions below 1:10,000,000 had little effect on the performance of starter HP in the cheese vat, and that concentrations of 1:1,000,000 to 1:400,000 gave the most useful range.

Each day the pasteurizer, cooler, vats and equipment were washed thoroughly and treated with boiling water containing a chlorine disinfectant in order to reduce to a minimum the amount of phage infection from previous working.

Method of cheese manufacture

Milk of good quality was flash pasteurized at 155° F. and passed over an open vertical tubular cooler. The first 40–60 gal. from the cooler were rejected for the experiments, as it was found to contain sufficient phage to cause an appreciable development in the control vat in spite of the precautions taken to avoid contamination. The experimental milk taken immediately afterwards was run into two 80 gal. vats.

Vat 1 served as control in which phage contamination was kept as low as possible, while vat 2 was artificially infected with known amounts of hp phage contained in 100 ml. of sterile water, just before the addition of 1% starter HP. All care was taken to avoid contamination of the control vat, and the manufacturing procedure was kept as closely as possible the same in both vats up to the stage where phage began to have a noticeable effect, i.e. after 'drying'. Since it is the practice in commercial factories to hold 'slow' or 'dead' vats until the desired acidity is obtained, the processes of 'milling' and 'salting' were carried out when the acidity reached 0·70 and 0·80% respectively.

In this way cheese of the best quality obtainable under the conditions of the experiment was produced. The cheese were of export size (80 lb.) and were ripened in a curing room in the usual way. Manufacturing data are tabulated in Table 1, and acid production calculated as percentage lactic acid plotted against length of time from addition of starter is shown in Fig. 1.

Table 1. *Manufacture data*

80 gal. milk, 1% starter HP. Setting temperature 88° F., cooking temperature 100° F.
Vat 1 = 'control'; vat 2 = 'infected'; titre phage preparation employed 10^{-12}.

Date of manufacture ...	7. xii. 42		8. xii. 42		9. xii. 42		10. xii. 42		11. xii. 42	
Vat	1	2	1	2	1	2	1	2	1	2
Amount of phage added...	Nil	0·5 ml.	Nil	0·6 ml.	Nil	0·7 ml.	Nil	0·8 ml.	Nil	1·0 ml.
Parts phage/milk ...	—	1 : 800,000	—	1 : 700,000	—	1 : 600,000	—	1 : 500,000	—	1 : 400,000
Acidity at setting	0·14	0·14	0·145	0·145	0·15	0·15	0·14	0·14	0·14	0·14
Acidity at cutting	0·09	0·09	0·095	0·095	0·095	0·095	0·09	0·09	0·095	0·09
Time set-dry, hr. min.	2 55	2 55	2 55	2 55	2 55	2 55	2 55	2 55	2 55	2 55
Time dry-mill, hr. min.	1 55	2 25	1 35	2 0	1 35	2 50	1 40	3 40	1 40	4 35
Time mill-salt, hr. min.	0 50	1 0	0 40	0 55	0 40	1 10	0 35	2 0	0 50	3 5
Time dry-salt, hr. min.	2 45	3 25	2 15	2 55	2 15	4 0	2 15	5 40	2 30	7 40
Acidity at running	0·13	0·13	0·13	0·13	0·125	0·125	0·13	0·13	0·12	0·12
Acidity at drying	0·18	0·18	0·19	0·19	0·18	0·18	0·18	0·18	0·185	0·18
Acidity at milling	0·70	0·70	0·70	0·70	0·70	0·70	0·70	0·70	0·70	0·67
Acidity at salting	0·79	0·78	0·80	0·80	0·79	0·81	0·81	0·80	0·80	0·75
Phage titre; whey at running	10^{-1}	10^{-6}	10^{-2}	10^{-5}	10^{-2}	10^{-5}	10^{-1}	10^{-6}	10^{-2}	10^{-8}
Phage titre; cheese at 7 days	10^{-3}	10^{-9}	10^{-3}	10^{-11}	10^{-4}	10^{-12}	10^{-3}	10^{-13}	10^{-3}	10^{-14}
Cheese *p*H: 1 day	5·01	5·08	4·90	5·05	4·98	5·14	4·91	5·20	4·94	5·19
14 days	4·96	4·95	4·93	4·95	4·93	5·11	4·88	5·19	4·90	5·19

Observations on acid development and phage multiplication

Good acid development which varied little from day to day and resulted in a curd of good 'body', well cheddared at salting time, was obtained in all control vats. The cheese whey was never completely free from phage, so that a few plaques with the 10^{-1} and 10^{-2} dilutions were shown when the whey was tested at 'running'.

A similar rate of acid development was obtained in all vats up to 'drying', and in the infected vats a graded acid development occurred thereafter. Differences in acid production in milk containing 0·4–0·6 ml. of phage preparation were noticeable to the cheesemaker and resulted in differences in the condition of the curd as compared with that in the control vats. The effect of these amounts of phage showed slight variation when tested on different days. It was evident that these initial additions were just on the borderline in causing marked influence on the performance of the starter. The vat to

which 0·7 ml. phage was added became 'steady' from 0·50% acidity onwards, and the curd was slightly 'sweet' but fairly well cheddared. The addition of 0·8 ml. resulted in a definite slowing down between 'drying' and 'milling', while the vat with 1·0 ml. almost stopped at 0·50% acidity and was very slow in rising to the salting acidity. The curd was 'sweet' and very poorly cheddared. Phage multiplication in the infected vats was well marked, the whey at 'running' showing titres up to 10^{-8}. Thus when such titres are obtained in practice degrees of slowness occur which may be due to infection with phage and if so can be eliminated by a more thorough cleansing of vats and equipment, provided the starter is free from infection.

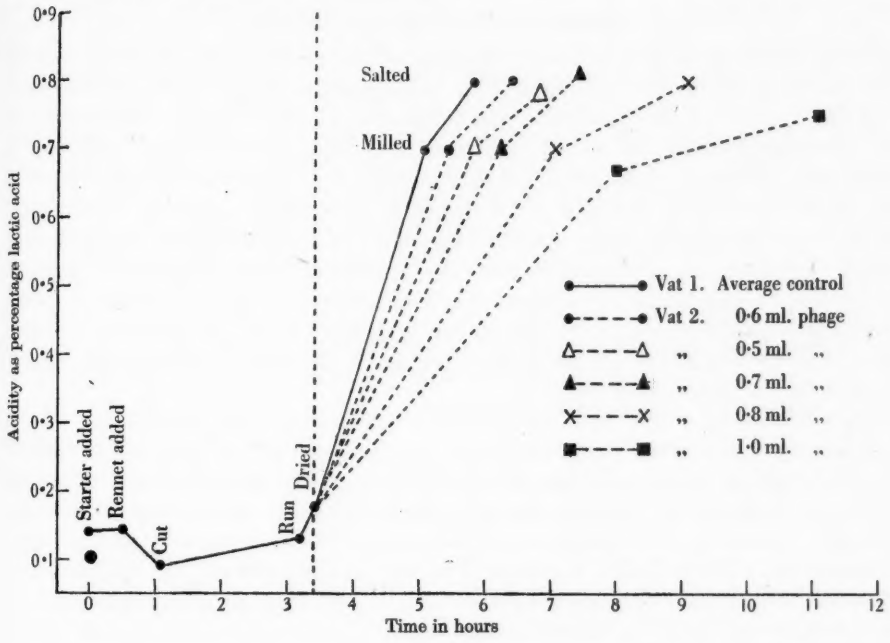

Fig. 1.

Examination of cheese

The phage content of the cheese was determined daily for 7 days by spotting on plates spread with the susceptible organisms, the dilution prepared from 1 g. of cheese in sterile water. Duplicates agreed very closely. No appreciable increase in strength occurred in any cheese after the first day, and in the experimental cheese the phage reached its maximum strength shortly before or after the curd was 'hooped'. High titres of 10^{-9}–10^{-14} were shown in the cheese made from artificially infected milk while the control cheese showed titres of only 10^{-3}.

The cheese were examined at 14 days and at 3 months.

Grading at 14 *days.* All the control cheese were of very good quality with smooth body and clean mild flavour. Cheese made from milk infected with more than 0·5 ml. of phage preparation showed marked differences in flavour and body which resulted in the loss of grade points. The cheese were 'sweet', and the higher additions of phage

gave cheese with a characteristic 'whey' flavour. The body of the experimental cheese ranged from slightly 'firm' to slightly 'curdy' through to 'mealy' and 'curdy'. None of the cheese showed defects of any type sufficient to warrant their relegation to 'second' grade at 14 days.

Grading at 3 months. The defects in cheese from milk infected with more than 0·6 ml. of phage preparation were more pronounced at maturity, and more than 0·7 ml. gave 'second' grade cheese with 'fermented' and 'yeasty' flavour and weak 'pasty' body. These cheese were also of very open texture owing to the development of fermentation and gas-producing organisms in the curd which had not reached a normal *p*H value.

Remarks on manufacture and grading

When acid production is obviously retarded within the first 3½ hr. of the process by the action of phage developed either from contamination in the starter itself or from a heavy infection of the cheese milk, 'dead' vats result with the consequent production of poor-quality cheese. In the above experiments designed to simulate conditions in commercial factories where occasional slow vats are experienced in the later stages of the process, the phage infection was adjusted to have effect only after 'drying', i.e. 3½ hr. after the addition of the starter to the milk. In the control vats, the total time required to reach the salting acidity (0·80%) was under 6 hr., while the extent to which the process was prolonged in the experimental vats depended upon the amount of phage infection originally present. If the process was prolonged beyond 7 hr., with the cessation of acid production before the *p*H of the green cheese was reduced below about *p*H 5·0, a second grade product resulted.

As will be evident from the results, there is a margin of safety for the rate of acid production, within which good commercial quality cheese can be produced in spite of the fact that an appreciable quantity of phage is present and multiplies during the manufacturing process. Factory managers frequently claim that certain single-strain cultures weaken in performance in the cheese vat after being in use for some time, although the cultures remain unaltered in activity at the Institute. An explanation of this possibly lies in the fact that phages to which the organisms are susceptible 'build up' in a factory and supply sufficient constant infection to the cheese milk to cause a comparative 'slowness' of the organisms in the vat. The rate of acid production would doubtless return to its maximum if the infection were eliminated.

Hence it is very probable that the so-called 'normal' performance of a particular starter varies considerably from factory to factory.

Discussion

The danger of contamination of starter cultures with phage and consequent failures in the bulk starter or in the cheese vat has been well recognized in commercial factories where 'single strain' starters have been in use continuously, but hitherto it has not been possible to view in true perspective the effect of phage contamination of vats and equipment. It is now evident that many instances of slowness in the later stages of the cheese-making process have been due in some measure to the rapid multiplication of phage which gained access to the milk from various sources. The variable conditions prevailing in practice with the use of pure cultures, either singly or in admixture, make it impossible to set any rigid dividing line or margin of safety. It is obvious, however,

that although care should be directed mainly towards protection of starter cultures during propagation, some attention must also be given to correct cleansing and disinfection of factory surroundings and equipment in order to avoid gross contamination. Clearly, also, a reduction in the initial infection received by the cheese milk, besides resulting in reduced time and labour costs and in the production of better quality cheese, will also greatly lessen the risk of starter failures due to contamination with air-borne phage derived from the cheese whey.

SUMMARY

The presence, in the cheese milk, of phage active against the organisms of the 'single-strain' culture in use as starter leads to its multiplication during the cheese-making process. The extent to which the presence of phage is made evident in the vat depends largely upon the amount of initial infection. A heavy infection permits sufficient multiplication of the phage to cause lysis of the organisms and consequent cessation of acid development before the manufacturing process is completed. A light infection may have no noticeable influence upon the performance of the starter in the vat. Intermediate amounts have an effect on the rate of acid development in the later stages of the process, but within certain limits there is little adverse influence on the quality of the cheese produced.

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312. THE PERSISTENCE AND RECOVERY OF BACTERIOPHAGE IN CHEESE

By AGNES A. NICHOLS and J. Z. WOLF,
National Institute for Research in Dairying, University of Reading

Introduction

During the cheese-making season of 1942 samples of commercial starters and of the corresponding samples of whey from cheese factories have been regularly examined for bacteriophage. Phage has been recovered from many of the whey samples and has frequently been shown to be the cause of starter failure. Occasionally, however, although phage has been suspected as being the cause of 'slowness' or starter failure it has not been possible to confirm this by applying an activity or phage detection test on milk as used in this country (1, 2), because when the report of 'slowness' has been received and a request for samples despatched, the original starter and corresponding sample of whey have no longer both been available. If these starter failures were due to phage it would be reasonable to suggest that it would persist and that it should be recoverable from the cheese itself.

Johns & Katznelson (3) appear to be the only workers who have reported attempts to recover phage from cheese. They failed to demonstrate the presence of phage after 18 days' ripening in cheese which had been made from milk in which the starter had been lysed by bacteriophage. They added decimal dilutions of the cheese to sterile milk tubes seeded with the original starter, but no lysis was obtained even in the lowest dilution (10^{-1}), and they suggested that the phage had died out during this period. They did not appear, however, to have prepared a cheese extract and to have passed it through a Seitz filter, and consequently it is possible that the phage-resistant streptococci present in the comparatively young cheese used may have masked the lytic effect of any phage present in the original starter.

Since it was possible to obtain samples of different batches of cheese which had been made from starter which had failed and of which the original mother starter was available, it was decided to investigate whether the phage could be recovered from the cheese and whether it persisted for any length of time.

Methods

The cheeses examined were obtained from three sources. The first source was a dairy school, from which two cheese were obtained. These cheese were made from milk in which the starter had been almost completely lysed by phage. The other sources were two factories, one of which was in Dorset and the other in Wales. The factory samples were of cheese, each one from many from the same vat which had shown a 'slowness' during the making of from 1 to 2½ hr. longer than the normal make. Sample bores of cheese were taken at intervals by using sterile circular tube 'borers'. The extract was prepared by weighing out approximately 20 g. of cheese which had previously been

cut up into small pieces into a sterile stoppered bottle which contained approximately 50 g. of glass beads. Quarter strength Ringer's solution was added to the cheese samples in the bottle in the proportion of 2:1 for the dairy school cheese, and of 3:1 for all the other samples. The mixture was thoroughly shaken and usually filtered through sterile glass-wool to remove some of the insoluble material, after which it was centrifuged in sterile tubes at 2000 r.p.m. for 5 min. The centrifuged extract was filtered through a Seitz filter and tested against strains which were components of the starter used for the original cheese.

Two methods were used to indicate the presence and concentration of phage:

(1) The first method was based on an activity test in milk. A 1% inoculum of some of the starter strains which were phage susceptible and which had been grown in milk for 18 hr. at 22° C. was added to 25 ml. of sterile milk in a screw-capped bottle. Decimal dilutions of the extract in $\frac{1}{4}$ strength Ringer's solution were made on the initial tests to 10^{-14}, but later as the range of phage recovery was known, a smaller number of dilutions was necessary. 1 ml. of each dilution was added to the inoculated milk and 1 ml. of $\frac{1}{4}$ strength Ringer's solution was added to the controls. The bottles were then incubated in a water-bath at 30° C. and shaken at regular intervals. 10 ml. of the milk after from 5 to 7 hr. incubation, according to the rate of acid production, were titrated with $N/10$ caustic soda. If the sample containing added extract gave an acidity of 8–10% less than that of the control, it was considered that phage had been present in the dilution of the extract which had been used. In this connexion a criticism might be raised that 'slowness' in the making was not due to the presence of phage but to other factors inhibitory to the growth of starter and that the quantitative assay in the cheese extracts reflects not the phage titre but the concentration of such factors. It is possible that phage and such inhibitory factors could operate concomitantly. This criticism does not apply to the cheese made at the dairy school where additional evidence is available to show that the milk was normal in respect of inhibitory factors. It is doubtful and very unlikely that an inhibitory factor could occur in the high concentration (10^{-7}) in which the phage titre has been found in these cheese. Thus Whitehead(4) has shown that a 3% concentration of inhibitory milk was necessary to demonstrate this phenomenon. More recent work in this country has indicated that such factors may inhibit up to the 10^{-4} dilution (5). Further, it has been possible to isolate the following twenty-one specific bacteriophages, i.e. B7/156/157/158, B25/156/157, B27/156/157, 278/1/10, 298/20, 299/1/2, 295/1/7/9/12/16/20, 303/4/11/12, by the process of picking isolated plaques from the wheys at the time of making the cheese. The high concentration in which the phage occurred accompanied by the isolation of individual plaques disposes therefore of much of this criticism. Further, from one cheese (fully reported in Table 3) it has been possible to obtain clear plaques from the dilute cheese extract 66 weeks after making, and had it been appreciated at the time it is felt that phage could have been demonstrable by plaques from the remaining cheese extracts themselves.

(2) Using yeast dextrose broth as a medium (2% peptone, 1% lemco, 0·5% dextrose, 0·5% salt, 0·3% yeastrel) tests were made using the clearing of the turbidity due to growth in broth as an indication of the presence of phage. Two drops or approximately 0·1 ml. of a 1/10 dilution in $\frac{1}{4}$ strength Ringer's solution of an 18 hr. yeast dextrose broth culture of the strain were added to 1 ml. of sterile broth contained in a small tube ($3 \times \frac{4}{10}$ in. internal diameter). Two drops, i.e. 0·1 ml. of the dilutions of extract used

in the milk activity tests, were then added. Control tubes were also set up but without the addition of extract. The tubes were pre-warmed for an hour at 37° C. and then incubated at 30° C. The tubes were shaken and examined for development of turbidity with subsequent clearing at hourly intervals during the day, i.e. for about 9 hr., and were then placed on the laboratory bench overnight and a final reading taken at 24 hr. In broth the phaging cultures were easy to interpret.

It should perhaps be mentioned that in the preliminary stages of this work the extracts were also tested directly in milk activity tests against the original starters. As might be expected, however, this was not such a sensitive indication of the presence of phage in the extract as when some of the phage-susceptible strains from these starters were used as host. There would be an additional disadvantage in the broth test if the starter itself were used instead of phaging strains derived from it. Since the starters were originally commercial mixtures of several strains it would be impossible to get complete clearing of the broth even if phage were present.

Results

A brief note of the persistence and recovery of phage in cheese has been made previously (6) but the full results are now presented in Tables 1–3. It will be seen that:

(1) It has been possible to demonstrate phage both in the two cheese made at the dairy school (Table 1) and in the cheese made on a large factory scale (Tables 2 and 3). Thus the phage has retained its virulence throughout in cheese from three distant localities and made from different starters.

(2) The figures given indicate to what extent the phage was present, for example, 10^{-4} indicates that phage was active in the 1/10,000 dilution. The milk activity test for these particular strains appears to be the more sensitive of the two tests used, the phage titre as shown by the broth test usually being lower. There were isolated samples which showed a phage concentration of 10^{-8} and 10^{-9}, but the highest dilutions of extract in which bacteriophage was most frequently detected were 10^{-6}–10^{-7}.

(3) There is very little change in titre of the phage during cheese storage. It should be noted, however, that the moisture content of cheese (especially of Derby and Cheshire, Table 1) will decrease on prolonged storage, and this may indirectly exert some influence on the titre figure of the phage.

(4) The bacteriophages examined persisted in the cheese for at least a year, and as cheese is seldom stored for much longer periods it is probable that phage will persist as long as the cheese itself.

Discussion

(1) The demonstration of phage in cheese may be rendered more sensitive if phage-susceptible strains obtained from the original starter are used as a host in preference to the starter itself.

(2) Bacteriophages which attack cheese starters are not destroyed by some of the agents which readily destroy the majority of bacteria. Thus such phages are resistant to the action of most of the common disinfectants (7). They are also heat resistant (8, 9, 10). Since in cheese the aqueous phase contains common salt in concentration of the order of 5%, the fact that phage can exist in this apparently unfavourable medium is another indication of the hardihood of phage.

Table 1. *Bacteriophage titre of extracts of two cheese made at a dairy school from milk in which the starter completely failed*

Cheese made on 2 May 1942

Age of cheese on testing weeks	Type of cheese	Starter strain number: B/7 Milk activity	B/7 Broth test	B/25 Milk activity	B/25 Broth test	B/27 Milk activity	B/27 Broth test	Type of cheese	Starter strain number: B/7 Milk activity	B/7 Broth test	B/25 Milk activity	B/25 Broth test	B/27 Milk activity	B/27 Broth test
15	Cheshire	—	—	10^{-5}	10^{-5}	—	10^{-4}	Derby	—	—	—	10^{-3}	10^{-7}	10^{-3}
20		10^{-4}	10^{-4}	10^{-5}	10^{-5}	10^{-6}	10^{-5}		10^{-4}	10^{-3}	10^{-5}	10^{-5}	10^{-5}	10^{-5}
26		10^{-4}	10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-5}		10^{-4}	10^{-3}	10^{-4}	10^{-5}	10^{-4}	10^{-5}
35		10^{-5}	10^{-4}	10^{-6}	10^{-5}	10^{-7}	10^{-6}		10^{-4}	10^{-3}	10^{-5}	10^{-5}	10^{-6}	10^{-6}
44		10^{-4}	10^{-4}	10^{-4}	10^{-5}	10^{-4}	10^{-4}		10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-5}
52		10^{-7}	10^{-5}	10^{-7}	10^{-6}	10^{-7}	10^{-6}		10^{-5}	10^{-5}	10^{-5}	10^{-6}	10^{-5}	10^{-6}

Table 2. *Bacteriophage titre of extracts of factory Cheddar cheese from two different vats (A and B) made on the same day*

Age of cheese on testing weeks	Date cheese made	Vat	Starter strain number: 278/1 Milk activity	278/1 Broth test	278/10 Milk activity	278/10 Broth test	298/20 Milk activity	298/20 Broth test	299/1 Milk activity	299/1 Broth test	299/2 Milk activity	299/2 Broth test
11	16. vii. 43	A	10^{-3}	10^{-1}	10^{-3}	10^{-1}	—	—	—	—	—	—
		B	10^{-4}	10^{-4}	10^{-3}	10^{-1}	—	—	—	—	—	—
8	6. viii. 43	A	—	—	—	—	10^{-7}	10^{-4}	10^{-3}	10^{-5}	10^{-1}	10^{-4}
		B	—	—	—	—	10^{-3}	10^{-1}	10^{-5}	10^{-3}	10^{-5}	10^{-3}

— = no test.

Table 3. *Bacteriophage titre of extracts of factory Cheddar cheese from the 'slowest' vats from six different days' make*

Age of cheese on testing	Date cheese made	Vat no.	Starter strain number											
			295/1		295/7		295/9		295/12		295/16		295/20	
			Milk activity	Broth test	Milk activity	Broth test	Milk activity	Broth test	Milk activity	Broth test	Milk activity	Broth test	Milk activity	Broth test
weeks														
3	2. viii. 42	13	10^{-6}	10^{-3}	10^{-6}	10^{-4}	10^{-4}	10^{-3}	10^{-6}	10^{-4}	10^{-6}	10^{-4}	10^{-5}	10^{-3}
11			10^{-5}	10^{-3}	10^{-6}	10^{-3}	10^{-4}	10^{-1}	10^{-5}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-1}
18			10^{-5}	10^{-1}	10^{-4}	10^{-1}	10^{-5}	10^{-3}	10^{-5}	10^{-3}	10^{-5}	10^{-1}	10^{-4}	10^{-1}
26			10^{-5}	10^{-1}	10^{-5}	10^{-3}	10^{-3}	10^{-1}	10^{-5}	10^{-3}	10^{-5}	10^{-3}	10^{-4}	10^{-3}
36			10^{-4}	10^{-3}	10^{-5}	10^{-1}	10^{-4}	10^{-1}	10^{-6}	10^{-3}	10^{-5}	10^{-3}	10^{-4}	10^{-1}
44			10^{-4}	10^{-3}	10^{-4}	10^{-3}	No test		10^{-4}	10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-3}
52			10^{-4}	10^{-3}	10^{-5}	10^{-3}	10^{-4}	10^{-1}	10^{-4}	10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-1}
21	4. viii. 42	12	10^{-7}	10^{-5}	10^{-7}	10^{-5}	10^{-4}	10^{-4}	10^{-6}	10^{-7}	10^{-7}	10^{-5}	10^{-5}	10^{-3}
10½			10^{-9}	10^{-3}	10^{-9}	10^{-3}	10^{-6}	10^{-4}	10^{-5}	10^{-4}	10^{-6}	10^{-5}	10^{-4}	10^{-1}
16			10^{-5}	10^{-2}	10^{-4}	10^{-3}	10^{-4}	10^{-3}	10^{-5}	10^{-3}	10^{-4}	10^{-1}	10^{-5}	10^{-3}
			303/4		303/11		303/12							
			Milk activity	Broth test	Milk activity	Broth test	Milk activity	Broth test						
2	7. viii. 42	9	10^{-4}	10^{-3}	10^{-1}	10^{-1}	10^{-7}	10^{-1}						
10			10^{-3}	10^{-1}	10^{-4}	10^{-1}	10^{-5}	10^{-1}						
17			10^{-3}	10^{-1}	10^{-3}	10^{-1}	10^{-3}	10^{-1}						
1½	10. viii. 42	2	10^{-1}	10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-1}						
10			10^{-4}	10^{-1}	10^{-3}	10^{-1}	10^{-4}	10^{-1}						
1½	11. viii. 42	4	10^{-6}	10^{-4}	10^{-5}	10^{-4}	10^{-7}	10^{-3}						
10			10^{-5}	10^{-1}	10^{-5}	10^{-3}	10^{-7}	10^{-3}						
1½	11. viii. 42	8	10^{-4}	10^{-4}	10^{-4}	10^{-3}	10^{-6}	10^{-3}						
10			10^{-5}	10^{-1}	10^{-4}	10^{-1}	10^{-4}	10^{-3}						
16			10^{-6}	10^{-3}	10^{-4}	10^{-3}	10^{-6}	10^{-1}						
26			10^{-5}	10^{-3}	10^{-5}	10^{-3}	10^{-5}	10^{-3}						
34			10^{-6}	10^{-1}	10^{-7}	10^{-3}	10^{-6}	10^{-1}						
42			10^{-4}	10^{-3}	10^{-6}	10^{-3}	10^{-6}	10^{-4}						
51			10^{-6}	10^{-1}	10^{-6}	10^{-3}	10^{-6}	10^{-4}						
1	12. viii. 42	2	10^{-4}	10^{-3}	10^{-4}	10^{-3}	10^{-6}	10^{-4}						
9			10^{-4}	10^{-1}	10^{-4}	10^{-1}	10^{-6}	10^{-3}						
15			10^{-4}	10^{-1}	10^{-3}	10^{-1}	10^{-5}	10^{-1}						

(3) As phage can persist for such long periods in cheese it can obviously be carried over from one season to the next, and there is a real danger of building up a strong phage concentration in the spring through contact with last season's cheese if the corresponding starters are used.

(4) By using the extract of cheese, during the making of which the starter failed and of which there is no longer a starter or whey sample available, it should be possible to obtain useful information regarding the phage relationship of any new starters which it is proposed to introduce into the factory after such starter failure.

SUMMARY

Several cheese made from milk after the starter had failed, or during outbreaks of 'slowness' at three different places, were found to contain bacteriophage, which it is now possible to isolate from cheese where the whey is no longer available. The concentration of phage in cheese was found to be fairly constant when tested periodically during one year. The significance of such persistence on phage dispersal, its reintroduction into the dairy, and carry-over from one season to the next is pointed out.

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313. BACTERIOLOGICAL STUDIES OF ROLLER-DRIED MILK POWDERS, ROLLER-DRIED BUTTERMILK AND OF ROLLER- AND SPRAY-DRIED WHEY

By CONSTANCE HIGGINBOTTOM, *The Hannah Dairy Research Institute, Kirkhill, Ayr*

An investigation into the bacteriology of spray-dried milk powders has recently been made at this Institute by Nichols (1). The present paper describes the results of a parallel investigation into the bacteriology of roller-dried milk powders. Opportunity has been taken to include an examination of samples of roller-dried buttermilk and of both roller- and spray-dried whey.

The dried products were derived from a representative group of factories in Great Britain. Nine factories supplied samples of roller-dried separated milk powder, three supplied roller-dried full-cream powder, three supplied roller-dried buttermilk and five supplied roller-dried whey. Samples of spray-dried whey were obtained from four factories. In all a total of some 400 samples were examined, each factory providing one or more samples at roughly weekly intervals. The sampling extended over two periods, i.e. August to November 1939 and July to September 1940.

It will be appreciated that in a wide survey of this nature it was not possible personally to supervise the sampling nor to check the day-to-day conditions of plant operation. Instructions regarding the technique of sampling were issued to all factories, and general details regarding the methods of plant operation were at the same time secured. Attempts were also made (not always with success) to ascertain by correspondence the reasons for any marked anomalies in the counts of individual samples. While therefore the results cannot be expected to provide information regarding the influence of individual factors on the bacteriological quality of the dried products, they do furnish a useful index of the general level of the plate counts and of the predominating bacterial flora, as well as an indication of the variations likely to be encountered between different plants and between week-to-week samples taken from the same plant.

Experimental methods

Sampling

Samples of the roller-dried products were collected direct from the rollers into 6 oz. sterile bottles with minimum contamination, though elaborate precautions were impracticable. The use of bottles with a neck only 1 in. wide involved some handling of the powder which normally peels off from the rollers in large thin sheets or, with dried whey, in a form resembling lumps of popcorn (whey 'rock'). A number of samples were also taken direct from the sifter or from the bag or bin which was filled from it. Most of the samples were taken 2–3 hr. from the start of the run.

From one factory (plant A) four samples of dried separated milk from each batch were taken (*a*) direct from the rollers, (*b*) from the fine siftings, (*c*) from the coarse siftings, and (*d*) from the powder obtained by grinding the coarse siftings. The coarse siftings, which were mainly overheated fractions, represented only about 1% of the

total output. From another factory (plant C) samples were taken early in the run (about 8 a.m.) and again 4 hr. later (about 12 noon). These two sets of samples were intended to supply information regarding the plate counts at different stages of manufacture and packing and at different periods in the day's run.

Most of the samples of roller-dried whey were taken direct from the rollers, but at two factories samples of accumulated rock, taken before and after grinding, were also examined. Samples of spray-dried whey were taken direct from the collector.

All powders were examined as soon as received, usually within 1–3 days of manufacture. Random samples were retained to determine any change in plate count on prolonged storage.

Methods of examination

The method employed in the reconstitution of the milk powder was that described by Nichols (1). Complete reconstitution was not possible with the roller-dried products, the solubility being only about 78–85%. With full-cream milk powder, and to some extent with dried buttermilk, there was some tendency for the fat particles to coalesce and form a thick surface layer if shaking was too vigorous or prolonged. Care was therefore necessary to ensure maximum reconstitution with minimum fat coagulation. The reconstituted milk containing 20 g. powder in 180 ml. water, represented approximately a 1:10 solution. Further dilutions were made as required, using quarter strength Ringer solution.*

The plate count was determined at 37 and 55° C. on milk agar (2). Plates were prepared in duplicate and incubated for 3 days.

The opacity of the medium and the presence of many undissolved particles of powder made the reading of the 1 ml. plates (which were most frequently employed owing to the low counts of the roller-dried products) extremely difficult. An illuminated counting chamber was found to be less accurate than ordinary daylight owing to the impossibility of differentiating insoluble particles of the powder from bacterial colonies. Counts were therefore made in daylight using a hand lens or microscope to distinguish doubtful colonies.

The use of alkaline dilutions has been suggested as a means of overcoming this difficulty. Prickett & Miller (3) recommended the use of $N/10$ lithium or sodium hydroxide. A few trials were made with separated milk, buttermilk and full-cream milk powder using $N/10$, $N/25$ and $N/50$ lithium or sodium hydroxide solutions. $N/50$ alkali had little effect and with $N/25$ alkali the effect was not sufficient to be useful.

$N/10$ lithium or sodium hydroxide produced greater uniformity in the reconstituted milk and consequently some improvement in the opacity of the medium. As compared with reconstitution in water, however, the alkaline solution also affected the plate count to a variable extent depending on the particular milk powder under examination. This may be due to differences in flora, but it was decided not to pursue the matter further and all results in this paper were obtained using water for reconstitution.

* The whey rock, after weighing out into a sterile Petri dish, was transferred to the bottle containing the water blank by means of a sterile spoon since the small pieces of rock were inclined to stick if a paper cone was used. Some samples were in lumps too large to enter the narrow mouth of the bottle in which case the rock was first broken up into suitable small pieces in a sterile mortar. Complete solution was always obtained with the dried whey and no difficulty was experienced in reading plates in the counting chamber in the usual way.

With practically all samples presumptive coliform tests were carried out on 1, 10 and 20 ml. portions of the reconstituted milk, 1 ml. portions were also added to litmus milk and incubated at 37 and 55° C. In addition, a few samples were examined for moulds and yeasts by incubation at 22° C. on beer-wort agar (*p*H 3·5).

Keeping quality tests were carried out at 60° F. and the results judged by the length of period from reconstitution until clotting occurred spontaneously or on heating.

Examination of the flora was made from representative milk-agar plates used for the counts. All the colonies from the whole (or a representative section) of a plate were picked off into litmus milk, their reaction to this medium and their morphology noted, and a representative number examined in detail.

For the determination of heat resistance, 1 ml. of a 48 hr. litmus-milk culture was transferred to fresh litmus milk and held in a water-bath for the required time and temperature, 5 min. being allowed for the attainment of temperature.

Methods of tabulation

In analysing the plate counts logarithmic values have, for the reasons discussed by Robertson & Frayer (4) and Mattick, McClemont & Irwin (5), been used throughout. For tabulation it has appeared desirable to include not merely the mean log count and its standard deviation, but the equivalent geometric mean and the range covered by the geometric mean $\pm$ its standard deviation. This range, within which roughly two-thirds of all observed values fall, furnishes a far more useful indication of the normal limits of a series of plate counts than is provided by the extreme observed values, since the latter may include figures which are quite exceptional and unrepresentative. Thus a series of twenty-one plate counts at 55° C. which were made on samples of spray-dried whey at factory L gave a single exceptional count of 140,000 per g. Apart from this, only one value (a count of 7000) exceeded the maximum of the S.D. range, which worked out at 4260. Fifteen of the twenty-one counts, or almost exactly two-thirds, fell within the S.D. range of 70–4260 per g., while the remaining four counts fell between 20 and 60 per g. Such observations fully justify the employment of the S.D. range as the most practical measure of normal values for use in general surveys such as that described in the present paper. The value of the S.D. range has been stressed by Robertson & Frayer (4). It should be noted that, since the standard deviations are based on logarithmic values, they cover geometric and not arithmetic ranges; e.g. a standard deviation of, say, log 0·90, corresponds to a S.D. range extending from one-eighth of the mean to eight times the mean.

In tabulating geometric means and S.D. ranges, the values have been expressed in convenient round figures, since at best they only represent approximations.

Results and discussion

I. *Bacteriological quality*

(*a*) *Comparison of roller- and spray-dried products*

Table 1 gives a general summary of the plate counts at 37 and 55° C. for all samples examined. For comparison the results recorded by Nichols (1) for spray-dried milk powders have been included.

The outstanding feature in this table is the marked difference in the ranges of counts between the roller- and spray-dried products. Thus at 37° C. less than 5% of the counts

of the roller-dried products were above 100,000 per g., whereas from 70 to 85% of those of the spray-dried products exceeded this figure.* The differences at 55° C. are not so marked, but taking 10,000 per g. as the dividing line, less than 15% of roller-dried samples were above this level, in comparison with 45–55% for spray-dried samples. The mean values show comparable differences, those for the roller-dried products all lying below 1000 per g., while those for the spray-dried products approximated ½–2½ million per g. at 37° C. and 10,000–30,000 per g. at 55° C.

Similar differences are noted in the comparison, shown in Table 2, of the percentage of positive coliform tests found for roller-dried and spray-dried products respectively: the spray-dried whey gave over three times as many positives at all dilutions as did the roller-dried whey.

(b) Comparison of dried milks and dried whey

The figures summarized in Table 1 indicate that the differences between the levels of the plate counts of dried milk and of dried whey varied according to the variety of powder. With roller-dried products the plate counts of the dried whey at 37° C. were slightly but not significantly lower than those of the dried milks. The more extended frequency distributions and the higher S.D. both indicate, however, that the dried whey figures were subject to greater variability. A similar increase in variability was shown in the plate counts at 55° C., but at this temperature the mean count for the dried whey was above that for the dried milks. These results indicate a greater survival of thermophilic and a smaller survival of mesophilic bacteria in the samples of roller-dried whey, a result which might be anticipated in view of the more variable flora of the liquid whey and the more prolonged heat treatment to which it is subjected during drying.†

With the spray-dried products, where the heat treatment during processing is far less drastic, the dried whey showed significantly higher counts and higher variabilities at both 37 and 55° C., the geometric mean being three to six times that of the dried milks and the average S.D. representing a thirty-fold as against a seven-fold difference between the means and extremes. The counts for the samples of spray-dried whey at 55° C. showed a particularly wide distribution of values, the extreme range of observed counts extending from 20 to 140,000,000 per g., and the S.D. range from 600 to 1,380,000 per g. The fact that the frequency curve for this series gave two maxima indicates that plants of at least two distinct characters contributed to the results, a conclusion which will be shown later to be correct.

It will be seen from the footnote to Table 1 that the samples of dried milks consisted chiefly of dried separated milks, but that samples of dried full-cream milk and dried buttermilk were also included. The figures in the upper portion of Table 5 provide a basis for comparing the counts of these three products. The mean values for the counts at 37° C. show that there was no appreciable difference between the samples of dried separated milk and dried buttermilk when these were taken direct from the rollers, i.e. had undergone minimum handling. The dried full-cream samples gave a significantly higher mean value, but reference to the original data shows that these samples were

* It may be noted that an extensive series of tests on spray-dried milk powders carried out in 1942 showed a marked improvement in bacteriological quality over the results obtained in 1937–8 by Nichols(1).

† See, for instance, the article by Waite(6) who describes the formation of 'roller masses' of dried whey during drying.

Table 1. *Summary of plate counts of roller- and spray-dried products (all samples)*

Type of powder	No. of samples	Percentage distribution of log plate counts: Below 1·00	1·00 to 1·99	2·00 to 2·99	3·00 to 3·99	4·00 to 4·99	5·00 to 5·99	6·00 to 6·99	7·00 to 7·99	Above 8·00	Mean log count	S.D.	Geometric mean	S.D. range*
At 37°C.														
Roller-dried: Milk†	256	—	10	49	31	9	1	—	—	—	2·90	0·81	795	125– 5,130
Whey	64	—	25	47	11	13	4	—	—	—	2·78	1·06	600	50– 6,920
Spray-dried: Milk‡	388	—	—	—	10	19	30	34	6	1	5·65	0·82	446,700	67,600– 2,950,000
Whey	60	—	—	—	4	13	17	33	20	13	6·41	1·23	2,570,000	151,400–43,650,000
At 55°C.														
Roller-dried: Milk†	256	4	21	57	16	2	—	—	—	—	2·36	0·72	230	45– 1,200
Whey	64	6	8	41	31	11	3	—	—	—	2·86	1·00	725	70– 7,240
Spray-dried: Milk‡	254	—	2	18	34	30	15	1	—	—	3·94	0·84	8,710	1,260– 60,260
Whey	60	—	7	18	17	12	27	15	3	1	4·46	1·68	28,840	600– 1,380,000

* I.e. mean count ± standard deviation, which indicates the range within which two-thirds of the counts should normally fall.

† Comprises 214 samples of dried separated milk, 24 samples of dried full-cream milk and 18 samples of dried buttermilk.

‡ Data calculated from paper by Nichols(1).

Table 2. *Presumptive coliform test—all samples*

Type of powder	No. of samples	Percentage positive in: 1 ml.	10 ml.	20 ml.
Roller-dried separated milk	239	7	25	35
Roller-dried full-cream milk	25	—	—	4
Roller-dried buttermilk	16	—	19	19
Roller-dried whey	44	7	14	19
Spray-dried whey	60	23	66	66

Table 3. *Effect of conditions of plant operation on the plate counts of roller- and spray-dried products*

Type of powder	Plant	No. of samples	Plate count at 37°C.: Mean log count	S.D.	Geometric mean	S.D. range		Plate count at 55°C.: Mean log count	S.D.	Geometric mean	S.D. range	
Roller-dried separated milk (direct from rollers)	A, P, S	31	2·37	0·62	235	55–	975	1·73	0·41	55	20–	140
	B, E, G	15	3·57	0·73	3,710	690–	19,950	2·09	0·75	125	22–	690
Roller-dried separated milk (sifted and/or bagged)	A, P, S	31	2·56	0·91	365	45–	2,950	2·01	0·59	102	26–	400
	B, E, G	15	3·63	0·89	4,260	550–	33,110	2·27	0·55	185	52–	660
Roller-dried whey (direct from rollers)	W	8	1·93	0·56	85	25–	310	1·65	0·50	45	15–	150
	M	9	2·15	0·88	140	20–	1,070	2·98	0·67	950	205–	4,470
	C	15	2·71	1·02	510	50–	5,370	2·80	0·76	630	110–	3,630
	D	12	3·71	1·31	5,130	250–	105,000	3·12	1·45	1,320	45–	37,150
Spray-dried whey (direct from collector)	K	6	4·55	0·85	35,500	5,010–	251,000	4·03	0·10	10,700	8,510–	13,490
	R	15	5·46	0·94	288,400	33,100–	2,512,000	5·16	1·37	144,500	6,160–	3,388,000
	X	18	6·79	0·53	6,166,000	1,820,000–	20,890,000	6·04	0·68	1,096,000	22,910–	5,248,000

derived from plants which also gave exceptionally high counts at 37° C. with separated milk. Any differences between the counts of the three products at 37° C. can therefore be attributed to individual plant variations (see below). On the other hand, the significantly higher mean value for the dried buttermilk counts at 55° C., combined with the higher S.D., can hardly be accidental; it is probably attributable to the same causes as those operating in the case of dried whey, i.e. a more mixed initial flora and a more prolonged or drastic heat treatment.

As regards presumptive coliform tests (Table 2), differences were observed in the percentage of positive tests between the four roller-dried products. No explanation can be advanced to account for these differences, and it seems likely that they are due to purely random causes.

(c) *Differences between individual plants*

Nichols (1) found marked differences between the plate counts of samples derived from the various plants included in her survey of spray-dried milks. An examination of the results of the present investigation reveal similar differences between plants. Typical figures are given in Table 3. As regards roller-dried products, plants A, P and S (the results from which have been combined to furnish adequate figures) showed significantly lower means and ranges of counts ($P=0{\cdot}01$) at 37° C. than plants B, E and G. The counts at 55° C. showed differences in the same direction, though the means did not differ significantly ($P=0{\cdot}10$–$0{\cdot}25$). The differences in the 37° C. counts are most clearly demonstrated by examining the comparative numbers of samples falling within certain arbitrarily selected limits. Thus 94% of the samples from plants A, P and S gave counts below 10,000 per g., while 81% fell below 1000 per g. The equivalent figures for plants B, E and G were only 60% below 10,000 per g. and 23% below 1000 per g. Plant S gave particularly low and consistent counts. Out of a total of thirty-two samples received from this plant,* twelve were below 100 per g. and twenty-seven below 1000 per g.: only three counts exceeded 2000 per g. At the other extreme were the counts from plant G. A total of twenty-seven samples were received from this plant,* ten of separated and seventeen of full-cream milk powders. Of these, none gave counts below 100 per g., only eight were below 1000 per g., and fifteen exceeded 2000 per g.—three being over 40,000 per g.

As regards presumptive coliform tests, similar plant differences were apparent (top half of Table 4). Plants A, P and S gave consistently fewer positives at all three levels of testing than did plants B, E and G.

Variations in plate counts were also shown by the plants producing roller-dried whey (Table 3). Ten of the seventeen samples from plants W and M gave counts at 37° C. below 100 per g., and only one sample exceeded 600 per g. In comparison only four of the twenty-seven counts on samples from plants C and D fell below 100 per g., while fourteen exceeded 600 per g. and eight exceeded 10,000 per g., the highest observed count being 294,000. The level of the counts at 55° C. ran roughly parallel with those at 37° C.

The variations in the plate counts from the different spray-drying plants were also marked, as is again shown in Table 3. The small number of samples from plant K gave remarkably consistent and reasonably low figures: at 37° C. all but one fell below 100,000

* Comprising both direct and sifted samples, and including a few samples for which comparable counts at 55° C. were not available, and which were not therefore incorporated in Table 4.

Table 4. *Effect of conditions of plant operation and of handling on presumptive coliform tests of roller-dried separated milk*

Plant	Treatment	No. of samples	Percentage positive in 1 ml.	10 ml.	20 ml.
A, P, S	Direct from rollers	31	Nil	Nil	3
B, E, G	Direct from rollers	15	7	20	33
	Total	46	2	6	13
A, P, S	Sifted or bagged	31	10	16	19
B, E, G	Sifted or bagged	15	13	60	60
	Total	46	11	30	33
A	Direct from rollers	10	Nil	Nil	10
	Fine siftings	10	10	20	30
	Coarse siftings	10	60	70	70
	Powder from coarse siftings	10	40	80	80

Table 5. *Effect of handling on the plate counts of roller-dried products*

Type of powder	Treatment	No. of samples	Plate counts at 37°C. Mean log count	S.D.	Geometric mean	S.D. range	Plate counts at 55°C. Mean log count	S.D.	Geometric mean	S.D. range
Separated milk	Direct from rollers	87	2·73	0·77	535	90– 3,160	2·14	0·78	140	23– 830
	After sifting and/or bagging	87	2·91	0·91	815	100– 6,610	2·32	0·70	210	42– 1,050
Full-cream milk	Direct from rollers	12	3·16	0·86	1,450	200–10,470	2·30	0·53	200	60– 675
	After sifting and/or bagging	12	3·34	0·66	2,190	480–10,000	2·33	0·50	215	67– 675
Buttermilk	Direct from rollers	9	2·75	0·64	560	130– 2,450	3·07	0·46	1,175	410– 3,390
	After sifting and/or bagging	9	3·21	0·70	1,620	325– 8,130	3·02	0·39	1,045	425– 2,570
Separated milk (plant A)	Direct from rollers	10	2·17	0·29	150	75– 290	2·42	0·62	260	63– 1,100
	Fine siftings	10	2·28	0·43	190	70– 515	2·50	0·63	315	75– 1,350
	Coarse siftings	10	3·08	1·16	1,200	85–17,500	2·88	0·76	760	130– 4,360
	Powder from coarse siftings	10	3·26	0·49	1,820	590– 5,620	2·90	0·46	795	275– 2,290
Whey (plant M)	Direct from rollers	9	2·15	0·88	140	20– 1,070	2·98	0·67	950	205– 4,470
	Accumulated	9	3·06	0·91	1,150	140– 9,330	3·41	1·03	2,570	240–27,540
	Ground	9	2·94	0·42	870	330– 2,290	3·09	0·61	1,230	300– 5,010
Separated milk (plant C)	Direct from rollers, 8 a.m.	10	2·82	0·47	660	225– 1,950	2·65	0·85	445	60– 3,230
	Direct from rollers, 12 noon	10	2·72	0·54	525	150– 1,820	2·37	0·31	235	115– 480

per g. The samples from plants X and L showed a similar consistency, but at the opposite extreme of efficiency: only one out of the thirty-nine samples examined showed a count of less than 1,000,000 per g., and seven of the counts exceeded 100,000,000 per g. Plant R (which was identical in construction with plant X) gave variable figures, with an extreme range of observed values of from 12,800 to 122,000,000 per g. All but two of the samples were, however, below 1,000,000 per g., and eight out of the fourteen examined were below half this figure.

The counts on the spray-dried whey at 55° C. showed even more striking differences. Plant K again gave consistently low figures. The best counts were, however, given by plant L, with the remarkably low mean of 540 per g., an extraordinary finding in view of the phenomenally high counts at 37° C. The counts at 55° C. on the identical plants R and X were, on the other hand, exceptionally high, twelve of the thirty-three samples examined showing figures of over 1,000,000 per g., the highest for the two plants being 58,000,000 and 140,000,000 respectively. The practice adopted in these plants was to hold the whey at a relatively high temperature (*ca.* 160° F.) for periods of several hours before drying, a procedure which would be expected to lead to an extremely high thermophilic count in the powder.

As noted in the introduction, it has in most instances been impossible to determine the causes of the plant differences referred to in the above discussion. It is clear, however, that in the operation of many of the plants there is considerable room for more consistent and improved technique.

(d) Differences due to handling

It will be seen from the results for the roller-dried separated milks recorded in Table 3 that there was a small but consistent difference between the plate counts of samples taken direct from the rollers and those taken after sifting or bagging. A more extensive series of results on the effect of handling on the plate counts is given in Table 5. It is clear that with roller-dried products sifting and bagging involve an increase in the plate counts at both 37 and 55° C. of about 50%, though the results at 55° C. are somewhat variable. More exposed and prolonged handling involves markedly greater increases in the counts. Thus the coarse siftings of dried separated milk from plant A, and the accumulated dried whey from plant M gave mean counts which showed an eight- to ten-fold increase at 37° C. and a two- to three-fold increase at 55° C. over the fresh product taken direct from the rollers.

These general findings are confirmed by the results of the presumptive coliform tests recorded in Table 4. The percentages of positive tests are markedly higher for the sifted or bagged samples than for the samples taken direct from the rollers at all three levels of testing. Still higher percentages are given by the coarse siftings from plant A and by the samples of powder from these coarse siftings.

Such results indicate the need for scrupulous care and cleanliness in the handling of the dried products if consistently low counts are to be maintained in the finished product.*

* This point is well illustrated in the comment from one plant which showed exceptionally large differences between samples taken direct from the rollers and after sifting, in two instances increasing from about 100 per g. to over 50,000 per g.: 'We are not surprised at these results, as the powder has a considerable distance to travel before it passes through the sifter, *and as this track is totally enclosed it does not allow for any cleaning or sterilising.*'

As regards the counts at different periods of the day's run, the number of observations is limited, but they indicate (plant C, Table 5) that with roller-dried products this factor is unlikely to be a significant source of variation.

(*e*) *Mould counts*

It has been suggested that the number of mould spores present in the powder may be taken as an index of the extent of recontamination which has taken place subsequent to drying. To test this assumption seventeen samples of roller-dried separated milk were examined for moulds immediately after drying (i.e. direct from the rollers) and again after sifting or bagging.

Of these seventeen samples, nine which had been taken direct from the rollers gave mould counts exceeding 10 per g., against 11 of the sifted samples. Seven of the samples showed increased mould count after sifting, seven gave the same figures for the 'direct' and sifted samples, and three showed a decrease after sifting. Comparing the bacterial (as distinct from the mould) counts of these samples, the seven which showed an increase in mould count after sifting showed a three-fold increase in bacterial count; the seven which had identical mould counts before and after sifting showed a two-fold increase in bacterial count; and the three which showed a decrease in mould count after sifting also gave a three-fold *increase* in bacterial count. It seems very doubtful, therefore, whether an increase in the mould count, unless it is very marked, is likely to provide a reliable indication of recontamination subsequent to drying. This conclusion is borne out by a comparison of the mean bacterial counts corresponding to certain ranges of mould counts as shown in the following summary:

Range of mould counts per g.	No. of samples	Corresponding mean bacterial count, per g.
Under 10	14	890
10–90	14	1,050
100 or over	6	26,000

It will be seen that only when the mould count reached a figure of 100 or more was there a significant increase in the bacterial count. Moreover, two of the six samples with mould counts exceeding 100 were taken *direct from the rollers*, while on the other hand one of the sifted samples showed a mould count of less than 10 per g. and yet had a bacterial count of 6300 in comparison with an initial count on the 'direct' sample of only 130 per g. Such facts cast still further doubt on the view that mould counts may furnish a valuable index of recontamination.

(*f*) *Relation of plate counts to acidity of dried whey*

Since the acidity of milk products depends largely on the extent of bacterial multiplication, it appeared desirable to ascertain whether any correlation existed between the acidity of the samples of roller- and spray-dried whey and the plate counts at 37° C.

The acidity of the samples was determined by titration and potentiometrically. As would be expected, the titratable acidity and the *p*H values ran roughly parallel, the mean figures being as follows:

Type of whey powder	No. of samples	Titratable acidity		*p*H		
		Mean	S.D.	Mean	S.D.	*r*
Roller-dried	43	2·89	0·58	5·86	0·27	−0·45
Spray-dried	49	1·45	0·78	6·52	0·58	−0·78

It will be noted that the roller-dried products showed a markedly higher general level of acidities.

Contrary to expectation, no correlation was found between the acidities and the plate counts of either the roller- or spray-dried samples. Tables 6 and 7 give the relevant figures for plate counts versus titratable acidities. All that can be said is that with the roller-dried whey the majority of the counts fall below 1000 per g. and have an acidity above 2·0, whereas with the spray-dried whey the majority fall above 100,000 per g. and have an acidity below 2·0; otherwise the distribution is of a more or less random nature. A comparison of the plate counts with the *p*H values shows a similar lack of correlation. It is possible that this lack of correlation is attributable to the neutralization of the whey before drying, and/or to the addition of small quantities of formalin, which is not uncommonly used to prevent undue acidity development.

Table 6. *Relation of plate counts to titratable acidity (roller-dried whey)*

Titratable acidity	log plate count at 37° C.					
	1·0–1·9	2·0–2·9	3·0–3·9	4·0–4·9	5·0–5·9	Total
1·0–1·9	—	—	—	—	1	1
2·0–2·9	5	12	2	4	1	24
3·0–3·9	7	5	2	—	1	15
4·0–4·9	2	1	—	—	—	3
Total	14	18	4	4	3	43

Table 7. *Relation of plate counts to titratable acidity (spray-dried whey)*

Titratable acidity	log plate count at 37° C.						
	3·0–3·9	4·0–4·9	5·0–5·9	6·0–6·9	7·0–7·9	8·0–8·9	Total
Below 1·0	—	3	7	8	5	2	25
1·0–1·9	—	—	—	9	4	2	15
2·0–2·9	1	1	—	2	1	2	7
3·0–3·9	1	—	1	—	—	—	2
Total	2	4	8	19	10	6	49

(*g*) *Relation of plate counts at* 37° C. *to those at* 55° C.

The relation between the plate counts at 37° C. and those at 55° C. differs markedly according to both the process of manufacture and the nature of the product. With roller-dried milks the counts at 37° C. are on the average about three times those at 55° C. (Table 1), though the ratios vary with individual plants (Table 3). With roller-dried wheys, on the other hand, the counts at 55° C. actually exceed those at 37° C. (Table 1), though the ratios again vary with individual plants (Table 3). Roller-dried buttermilks fall intermediately.

With spray-dried products the differences are far more marked, the counts at 37° C. always being many times higher than at 55° C. This is shown clearly in Table 1, where the counts at 37° C. for both spray-dried milks and spray-dried wheys are from fifty to one hundred times as large as the counts at 55° C.* It is shown even more clearly from an examination of the counts of individual samples of dried whey. Out of the sixty samples of spray-dried whey examined, only eight showed a higher count at 55 than at 37° C.: on the other hand, out of sixty-four samples of roller-dried whey, forty gave a significantly higher count at 55 than at 37° C.

* Owing to the improved 37° C. counts of the spray-dried milk powders examined in 1942, this ratio was substantially lower in the latter year.

There is little doubt that the relatively higher thermophilic counts of the roller-dried wheys are due to the prolonged preheating, which would encourage the multiplication of thermophilic types. With the spray-dried wheys, on the other hand, the temperature of preheating will usually be too low to encourage the growth of thermophilic types. Spray-drying plants R and X, in which the milk was held at 160° F. for prolonged periods, form an exception to this general rule.

(*h*) *Changes in plate counts on storage*

The changes in the plate counts of the dried products during storage for periods up to one year are discussed in a separate paper(7). It may, however, be noted that most samples showed a material decrease in count within 3–6 months of manufacture, the maximum decrease varying with different products from 52 to 99%. The roller-dried products showed in general a more consistent mean decrease than the spray-dried products, while both roller- and spray-dried whey showed a more marked decrease than the corresponding milk powders.

II. *Keeping quality of reconstituted roller-dried milk*

An attempt was made to determine whether the keeping quality of the reconstituted roller-dried milks was directly related to the initial plate count of the dried powder. In the absence of any direct means of determining palatability and wholesomeness, the method employed in determining the keeping quality was the time to clot on boiling.

Table 8. *Relation of the plate counts of roller-dried powders to the keeping quality of the reconstituted milks*

Keeping quality days	Plate count at 37° C. per g. of powder					
	Below 2·00	2·01–3·00	3·01–4·00	4·01–5·00	Total samples	Percentage
Below 2½	2	7	2	—	11	14
2½–3	1	5	8	5	19	24
3½–4	3	18	10	4	35	45
4½–5	5	4	2	—	11	14
5½–6	—	2	—	—	2	3
Total	11	36	22	9	78	100

It will be seen from Table 8 that no relationship existed between initial plate count and keeping quality. This result might have been expected, since the drastic heat treatment to which roller-dried powders are subjected tends (as will be shown later) to reduce the varied flora of the original milk to a more limited number of specialized types, a considerable proportion of which are not acid producers (Table 10). It is clear, therefore, that the usual conception of keeping quality as applied to raw milks is not applicable to reconstituted roller-dried powders: indeed, tests based on time of clot on boiling may actually be misleading if used as a guide to the hygienic quality of a roller-dried powder.

III. *Bacterial flora of the dried products*

(*a*) *Litmus-milk reactions at* 37 *and* 55° C.

1 ml. portions of random samples of the reconstituted products were added to litmus milk and incubated for 5 days at 37 and 55° C. Table 9 shows that at both temperatures the predominating flora produced acid coagulation, with or without digestion, a reaction

which was particularly marked with the samples of spray-dried whey. The remaining reactions occurred with varying frequency.

A large number of representative colonies were picked off from plates prepared from the reconstituted samples, and were inoculated into litmus milk. Their reactions on incubation at 37 and 55° C. are summarized in Table 10. Acid-coagulating types still tended to predominate in all products except the roller-dried wheys at 37° C. and the

Table 9. *Litmus-milk reactions of original dried milk products (1 ml. reconstituted product to 9 ml. litmus milk)*

Type of powder	No. of samples examined	Percentage of samples showing: Acid coagulation with or without digestion	Neutral coagulation or digestion	Reduction of litmus	No change
Incubated at 37° C.					
Roller-dried separated milk	249	75	17	2	8
Roller-dried full-cream milk	26	81	19	—	—
Roller-dried buttermilk	17	82	18	—	—
Roller-dried whey	66	56	18	5	26
Spray-dried whey	59	100	—	—	—
Incubated at 55° C.					
Roller-dried separated milk	84	57	11	2	30
Roller-dried full-cream milk	14	72	14	—	14
Roller-dried buttermilk	11	100	—	—	—
Roller-dried whey	60	65	15	5	15
Spray-dried whey	47	92	—	—	8

Table 10. *Litmus-milk reactions of cultures picked from plates*

Type of powder	No. of samples examined	Percentage of samples showing: Acid coagulation with or without digestion	Neutral coagulation or digestion	Reduction of litmus or production of alkaline reaction	No change
Incubated at 37° C.					
Roller-dried separated milk:					
All samples	347	44	30	6	19
Direct from rollers	109	33	33	13	21
After sifting and/or bagging	97	27	32	7	27
Roller-dried full-cream milk	58	43	19	9	29
Roller-dried whey	50	28	26	12	35
Spray-dried whey	92	79	1	1	19
Incubated at 55° C.					
Roller-dried separated milk:					
All samples	245	63	7	5	25
Direct from rollers	46	43	7	7	43
After sifting and/or bagging	54	67	7	—	26
Roller-dried full-cream milk	46	4	35	—	61
Roller-dried whey	52	66	15	—	25
Spray-dried whey	61	69	7	5	19

full-cream milks at 55° C. The proportion of acid-coagulating types at 55° C. was, however, markedly higher than at 37° C., the only exception being the spray-dried wheys, where incubation at 37 and 55° C. both gave some 70–80% of these types. Only a small proportion of the cultures gave reduction without coagulation, or an alkaline reaction. With the roller-dried separated milks, for instance, only 1% gave reduction without coagulation and only 5% an alkaline reaction, while the figures for the roller-dried full-cream milk were 5 and 4% respectively. The number of cultures which produced no change in the

litmus milk remained fairly constant at 20–30% for all types of milk and at both temperatures of incubation.

It may be noted from Table 10 that at both 37 and 55° C. there were very marked differences between the flora of samples taken direct from the rollers and after sifting and/or bagging.

(*b*) *Cultures from plates incubated at* 37° C.

Of 422 cultures which were isolated from the roller-dried milks and examined in detail 215 (or 51%) were found to be micrococci or sarcinae, 162 (38%) were spore-forming bacilli, thirty-seven (9%) were non-spore-forming bacilli, and only eight (2%) were streptococci. In addition a thermoduric species of *Actinomyces* and an occasional yeast were found. Of the limited number of cultures isolated from roller-dried whey twenty-eight (56%) were spore-forming bacilli. The remaining flora included sarcinae, streptococci, non-spore-forming bacilli (*Achromobacter* sp.) and micrococci. The acid-coagulating cultures isolated from spray-dried whey consisted almost entirely of *Str. thermophilis* and *Str. faecalis*, the latter predominating. An occasional spore-forming bacterium was also isolated from spray-dried whey, and one sample contained a high proportion of a non-spore-forming bacillus, probably of the *Achromobacter* species.

The following brief notes may be made regarding the individual types of bacteria:

(i) *Micrococci and sarcinae.* From the 215 cultures noted above, forty apparently different strains were obtained, no one type occurring in predominant numbers. Of these forty strains, fourteen were classed as sarcinae and the remaining twenty-six as micrococci, the latter usually occurring in irregular masses. Pigmented forms were obtained in both groups. In litmus milk five of the sarcinae sp. and ten of the micrococci produced no change: the remaining twenty-five strains produced acid, usually with coagulation and digestion and with reduction of the litmus. No attempt was made to classify these strains, which varied markedly in their relation to gelatin, nitrate reduction and fermentation reactions. Heat resistance tests showed that only seven out of thirty-seven strains tested failed to survive exposure to 60° C. for 30 min.

(ii) *Spore-forming bacilli.* Classification of the 162 cultures of spore-forming bacilli isolated from roller-dried milk showed, on the broad lines suggested by Gibson(8), that the most frequently occurring types were *B. subtilis*, *B. megatherium*, and *B. cereus*, and less frequently *B. brevis* and *B. pumilus*. A few cultures were not classified. Only ten of the 162 cultures produced acid coagulation in litmus milk. These varied considerably in their biochemical reactions and have not been classified.

The twenty-six cultures of spore-forming bacilli isolated from roller-dried whey were of similar types to those isolated from the roller-dried milks.

All the spore-forming bacilli survived heating at 80° C. for 10 min., and a few which were tested at 90° C. for 10 min. also survived.

(iii) *Other types.* The non-spore-forming bacilli showed the characteristics of *Achr. liquefaciens* and *Achr. candicans*. The few streptococci isolated from the roller-dried products proved to be *Str. faecalis*: as already noted, this organism and *Str. thermophilis* were also found in spray-dried whey.

The thermoduric species of *Actinomyces* was obtained occasionally from several different sources, but its appearance was most marked for a period of 3–4 weeks at one plant. It grew equally well at 37 and 55° C. forming spreading chalk-white colonies. It showed

weak saccharolytic properties but was actively proteolytic, rapidly liquefying gelatin and digesting the neutral clot in litmus milk. Diastatic action was marked and there was no reduction of nitrate. Bernstein & Morton (9) describe a species isolated from pasteurized cheese, *Actinomyces casei*, which appears to give very similar reactions to those noted above but which differs in showing no hydrolysis of starch.

(c) *Cultures from plates incubated at* 55° C.

All the products examined gave similar types of flora on plates incubated at 55° C. The cultures causing neutral digestion or coagulation in milk were either typical strains of *B. subtilis*, or in a few instances the thermoduric species of *Actinomyces* already described. All the remaining cultures consisted of rods which produced swollen terminal spores and survived heating at 80° C. for 10 min. Those which produced acid coagulation with reduction of litmus did not usually grow at 37° C.; those which did not produce acid frequently grew well at 37° C. but less satisfactorily at 55° C. These thermophilic and thermoduric spore-forming bacilli showed considerable diversity in biochemical properties, and no attempt has been made to classify them in detail.

IV. *The grading of roller-dried milk products*

Following her survey of the bacteriological quality of spray-dried milks, Nichols (1) has suggested that the grading system devised by the American Dry Milk Institute (10) and operated in the United States might advantageously be adopted for spray-dried products in this country. The question whether it would be desirable to adopt a similar standard for roller-dried products naturally arises.

For two reasons it appears doubtful whether a suitable standard could meantime be formulated. In the first place the fact that the plate count decreases markedly during storage makes it difficult to fix an appropriate maximum without reference to the age of the sample: for instance, an old sample of initially poor bacteriological quality might well show a lower plate count than a fresh sample of relatively good bacteriological quality. In the second place there is increasing evidence that the conditions of testing which have hitherto been accepted may require considerable modification, in which case the fixing of a definite standard would clearly be premature. Nevertheless, certain points arise from the present work which have a general bearing on the subject and which merit brief discussion.

It is clear from the results recorded in Tables 3 and 5 that inefficiency in plant operation and in the methods of handling the dried product are reflected in markedly increased plate counts. Thus the counts for plants A, P and S usually fall within a few hundreds, while those of plants B, E and G run into several thousands (Table 3). Plate counts, even with the present technique, are therefore clearly of value in differentiating between the products of well and poorly managed plants.

It is, however, equally clear from Table 1 that standards suitable for spray-dried products would provide far too generous a latitude for roller-dried products. It is true that many of the counts of the spray-dried products included in Table 1 are exceptionally high, and would not be tolerated if a grading system were in force.* Nevertheless, even if the American standards were to be adopted, the maximum count for the highest

* See footnote to p. 311.

category, i.e. extra grade at 15,000 per ml. (corresponding to 150,000 per g. of powder), would be far too lenient to be adopted as a standard for roller-dried products.

Crossley(11) has suggested that a good quality roller-dried powder should not have a plate count of more than 20,000 per g. Examination of the original figures which form the basis of Table 1 shows that only 5% of the roller-dried samples examined in the present investigation exceeded this level. Indeed, it would appear from the results obtained with well-run plants that any standard which might be proposed for high quality roller-dried powders could justifiably be fixed at a fraction of the figure suggested by Crossley. Thus only 10% of the samples from plants A, P and S exceeded 5000 per g., and a standard of this level would therefore not appear to be unduly rigorous for a first-grade product.

Pending the formulation of a revised technique of testing and the subsequent accumulation of data on which a new standard can be based, the above figure might well be adopted as an interim standard for the highest quality of roller-dried powder. In view of the marked reductions in plate count with increasing age, it would, however, be necessary to stipulate a time limit within which the product should be examined.

Summary

1. Bacteriological studies have been made of some 400 samples of dried milk products comprising roller-dried separated milk, roller-dried full-cream milk, roller-dried buttermilk, roller-dried whey and spray-dried whey. The samples were derived from a total of plants operating throughout Great Britain.

2. The mean values for the plate counts at 37 and 55° C. of the roller-dried products were all below 1000 per g. as against ½–2½ million at 37° C. and 10,000–30,000 at 55° C. for the spray-dried products. The spray-dried products also gave three times as many positive presumptive coliform tests at all dilutions as did the roller-dried products.

3. The roller-dried whey and the roller-dried buttermilk both gave more variable counts at 37° C. and higher counts at 55° C. than the roller-dried separated milk and the roller-dried full-cream milk. The spray-dried whey gave higher and more variable counts at both 37 and 55° C. than the spray-dried milk.

4. Marked differences were observed in the plate counts of samples derived from different plants, some plants giving consistently low counts and others giving higher and more variable figures. Differences were also observed in the counts at different stages of handling.

5. Evidence was obtained which indicates that mould counts do not provide a reliable indication of recontamination subsequent to drying.

6. No correlation was found between the plate counts and the titratable acidity or *p*H of the samples of either roller- or spray-dried whey.

7. The relation between the plate counts at 37° C. and those at 55° C. differed according to the process of manufacture and the nature of the product. Prolonged preheating of the raw liquid prior to drying was assumed to be the cause of excessively high thermophilic counts.

8. No relationship was found between the keeping quality and the plate count of reconstituted roller-dried milks. It would appear that the measurement of keeping quality by time to clot on boiling is not applicable to such milks.

9. An examination of the reactions to litmus milk of the original dried products and of some 600 colonies picked from inoculated plates showed that acid-coagulating types predominated. This was particularly marked with the samples of spray-dried whey and with all samples incubated at 55° C.

10. Cultures from plates made from the roller-dried products after incubation at 37° C. consisted chiefly of micrococci, sarcinae and spore-forming bacilli, with a small percentage of non-spore-forming bacilli, chiefly *Achromobacter* species. A species of *Actinomyces*, streptococci and an occasional yeast were also found. The flora were markedly heat-resistant. Details of the classification of the cultures are given in the text.

11. Cultures from plates made from the spray-dried whey after incubation at 37° C. consisted almost entirely of *Str. thermophilus* and *Str. faecalis*.

12. All the products examined gave similar types of flora on plates incubated at 55° C. This consisted chiefly of spore-forming bacilli of two main types, which showed highly diverse biochemical properties. No attempt was made to classify these types in detail—typical strains of *B. subtilis*, and a thermoduric species of *Actinomyces* also occurred in small numbers.

13. The desirability of establishing bacteriological standards for roller-dried milk products is discussed. On the basis of the results recorded in the paper it is considered that a plate count not exceeding 5000 per g. would constitute a reasonable interim standard for high-quality products, pending the formulation of revised methods of testing.

The author wishes to express her thanks to the manufacturers of the roller- and spray-dried products for their co-operation in supplying the various samples and in furnishing details of the working conditions of the plants. The author also desires to express her appreciation of the interest and advice given by Dr N. C. Wright throughout the course of the work and in the collation of the results.

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637.143:576.8

314. THE EFFECT OF STORAGE ON THE PLATE COUNTS OF MILK AND WHEY POWDERS

By CONSTANCE HIGGINBOTTOM, *The Hannah Dairy Research Institute, Kirkhill, Ayr*

(With 4 Figures)

It is widely recognized that the plate counts of milk powders show definite although variable decreases during storage, but detailed figures illustrating the extent of the actual variations have seldom been recorded. The examination of milk and whey powders for other purposes has afforded an opportunity to collect such data. The results are presented in this paper.

The roller-dried milk products and dried whey were prepared and examined in 1939 and 1940 and the spray-dried milk powders in 1942. The powders were stored in rubber-stoppered glass bottles in subdued light or in the dark at room temperature and re-examined periodically. The count was determined in milk agar(1) plates incubated for 3 days at 37° C. and 2 days at 55° C. The method of reconstitution employed for the roller-dried powders and the dried whey has been described in a previous paper(2). A similar method was used for the spray-dried powders, but quarter strength Ringer solution replaced water for reconstitution and the degree of shaking was standardized at 50 excursions of about 1 ft. in 12 sec.

Results

The accompanying figures show the results in the form of scatter diagrams, decrease in log count being shown in the right-hand and increase in the left-hand squares. It should be noted that the scale used for the dried milks differs from that for the dried wheys.

From these figures the general tendency for the count to fall during storage is evident for both types of milk and whey powder, but it is equally clear that many samples showed little alteration in count after 6 months or even longer periods. A few samples even showed an apparent increase in count but, since the low moisture content of the powder would prevent any multiplication of the bacteria during storage, it may be assumed that such increased counts were due to the experimental error of the method of examination.

It will be noted that, although all types of powder showed considerable variation, the tendency to a decrease in count on storage was slightly more marked in the roller-dried than in the corresponding spray-dried products and was much more definite for whey than for milk powder. This, together with the variation in count, is illustrated in Table 1 where the percentages of samples falling within different ranges of alteration in count are given.

The differences in mean log count of 0·57–0·80 for spray-dried whey and of 1·21–1·44 for roller-dried whey show the greater tendency for the count of such products to fall off during storage as compared with the corresponding milk powders. Although over 40% of the milk powders stored for 3 months showed a decrease in count greater than 50%,

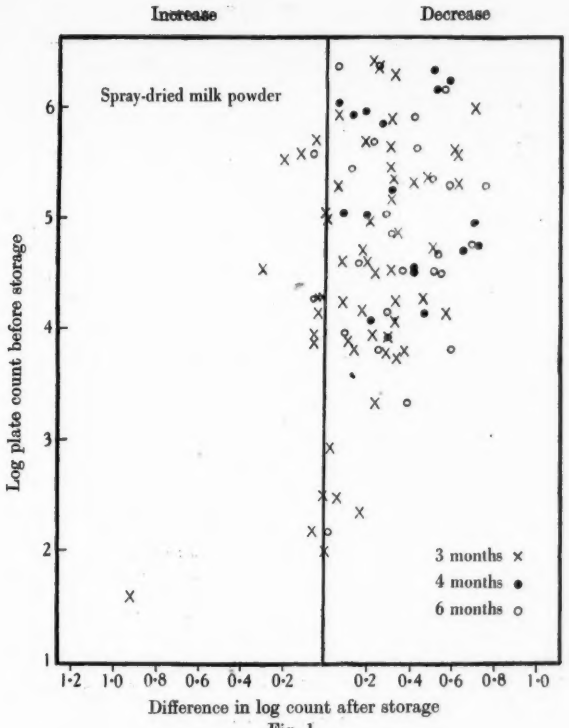
Increase
Decrease
Spray-dried milk powder
Log plate count before storage
6
5
4
3
2
1
3 months
4 months
6 months
1·2 1·0 0·8 0·6 0·4 0·2
0·2 0·4 0·6 0·8 1·0
Difference in log count after storage

Fig. 1.

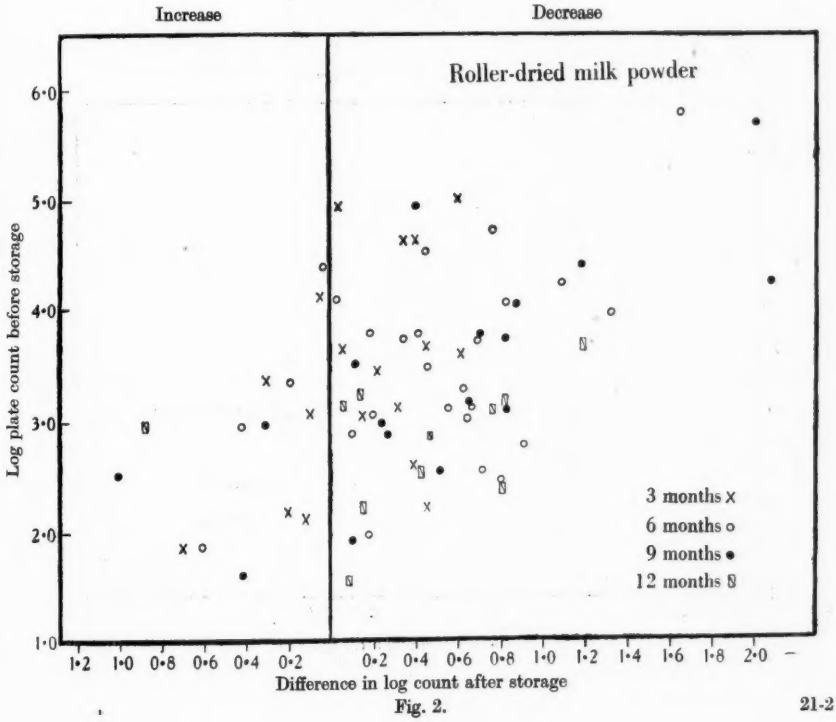
Increase
Decrease
Roller-dried milk powder
Log plate count before storage
6·0
5·0
4·0
3·0
2·0
1·0
3 months
6 months
9 months
12 months
1·2 1·0 0·8 0·6 0·4 0·2
0·2 0·4 0·6 0·8 1·0 1·2 1·4 1·6 1·8 2·0
Difference in log count after storage

Fig. 2.

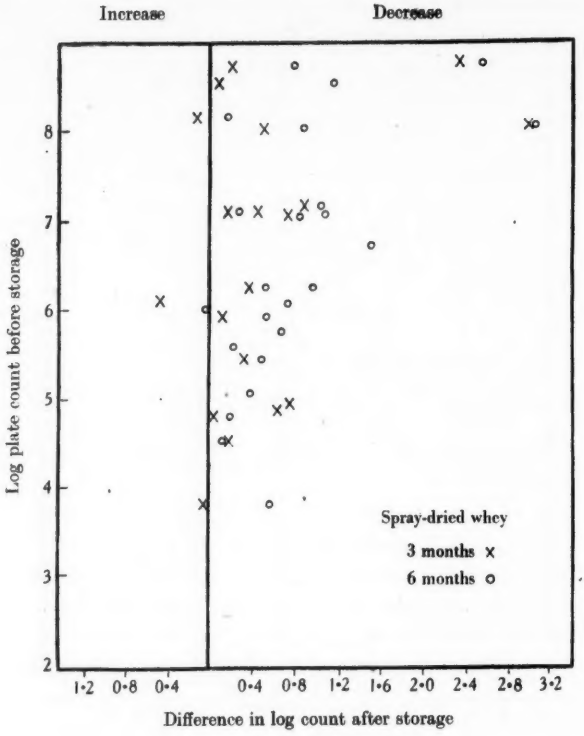

Fig. 3.

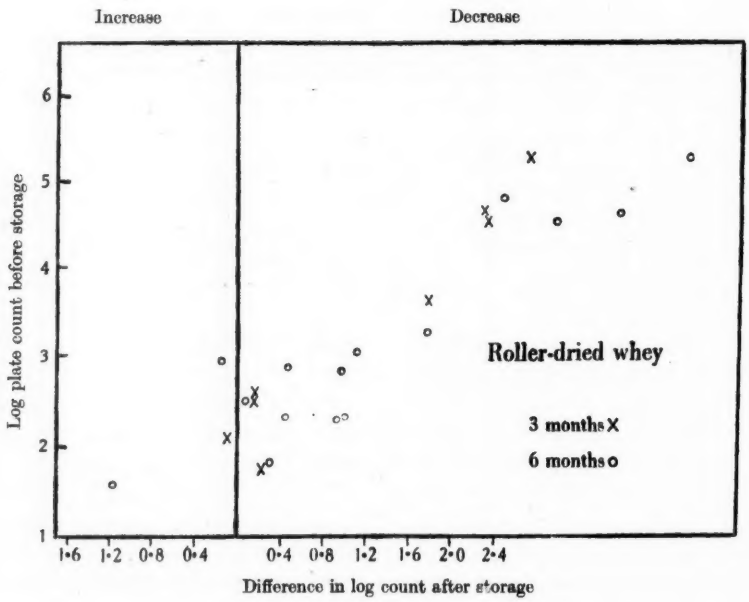

Fig. 4.

Table 1. *The effect of storage on plate count at 37° C.*

3 months				6 months				9 months		12 months	
Spray		Roller		Spray		Roller		Spray	Roller	Spray	Roller

Table 1. *The effect of storage on plate count at* 37° C.

	3 months				6 months				9 months	12 months
	Spray milk	Roller milk	Spray whey	Roller whey	Spray milk	Roller milk	Spray whey	Roller whey	Roller milk	Roller milk
No. of samples	56	18	19	8	27	27	23	16	17	11
Percentage giving:										
No alteration in count	5	—	—	—	—	—	—	—	—	—
<20% alteration	20	17	10	12½	19	7	4	6	—	—
20–50% decrease	27	11	22	37½	33	22	26	6	6	36
>50% decrease	41	44	58	50	48	60	70	75	76	55
>20% increase	7	28	10	—	—	11	—	13	18	9
Decrease in mean log count	0·19 (4·49–4·30)	0·17 (3·37–3·20)	0·57 (6·61–6·04)	1·21 (3·38–2·17)	0·33 (4·84–4·51)	0·44 (3·45–3·01)	0·80 (6·57–5·77)	1·44 (3·29–1·78)	0·70 (3·37–2·67)	0·36 (2·79–2·43)
% decrease in mean count	35	32	73	94	53	64	84	97	80	56

Table 2. *The effect of length of storage period on the plate count at* 37° C.

	Spray-dried milk powder (20 samples)		Spray-dried whey powder (17 samples)	
	3 months	6 months	3 months	6 months
Percentage giving:				
<20% alteration	10	20	12	—
20–50% decrease	30	35	23	27
>50% decrease	55	45	53	71
>20% increase	5	—	12	—
Decrease in mean log count	0·27 (4·72–4·45)	0·29 (4·72–4·43)	0·55 (6·80–6·25)	0·87 (6·80–5·93)
% decrease in mean count	46	49	72	90

Table 3. *The effect of storage on plate count at* 55° C.

	3 months				6 months				9 months	12 months
	Spray milk	Roller milk	Spray whey	Roller whey	Spray milk	Roller milk	Spray whey	Roller whey	Roller milk	Roller milk
No. of samples	48	18	19	16	26	29	20	19	17	12
Percentage giving:										
No alteration in count	9	5½	5	—	4	3	5	—	12	—
<20% alteration	8	28	27	31	27	31	15	5	—	25
20–50% decrease	31	28	31½	13	4	24	40	37	35	25
>50% decrease	19	5½	5	37	27	28	20	42	18	8
>20% increase	33	33	31½	19	38	14	20	16	35	42
Alteration in mean log count	−0·09 (3·25–3·16)	−0·09 (2·52–2·43)	+0·01 (4·16–4·17)	−0·12 (3·53–3·41)	−0·03 (3·24–3·21)	−0·21 (2·19–1·98)	−0·20 (4·66–4·46)	−0·23 (3·40–3·17)	−0·07 (2·60–2·53)	+0·13 (2·68–2·81)
% alteration in mean count	−19	−18	+ 2	−24	−7	−39	−35	−41	−15	+35

− decrease; + increase.

some 30–40% showed either an alteration of less than 20% or an apparent increase, and the mean decrease in log count was therefore only 0·17–0·19. After 6 months the mean decrease had risen to 0·33–0·44 but about 20% of all samples still showed either less than a 20% variation or an apparent increase in comparison with the original count.

The comparisons shown in Table 1 are, however, not entirely valid, since the individual samples examined after the varying periods of storage were not always the same. A fairer comparison is shown in Table 2, where the results for *identical* samples are shown after both 3 and 6 months' storage. The mean decrease in count of the spray-dried milk powders showed little alteration between 3 and 6 months: the spray-dried whey powders showed a more definite decrease in count, but this was still small in comparison with the magnitude of the original count.

The alterations in the counts at 55° C. (Table 3) were even more variable than those at 37° C., and the mean log counts showed no consistent trends during storage. It is, however, not surprising that significant differences could not be demonstrated in the thermophile counts, since the flora has been shown(2, 3) to consist almost entirely of spore-forming bacilli.

Summary and conclusions

It is evident from the foregoing results that decreases in count may occur during the storage of milk and whey powders. The extent of this decrease varies, however, with the type of powder and even between different samples of the same type. Further, many samples may show little or no alteration in the numbers of viable organisms surviving storage even for considerable periods.

Nevertheless, it is obviously important that for grading purposes or in relation to plant control a time limit should be fixed within which the plate count of any milk or whey powder should be determined. No data are available to show how rapidly organisms may die out during storage periods of less than 3 months, but it is tentatively suggested that all powders should normally be examined within, say, 14 days of manufacture. Unless such a limit is fixed, those samples in which the count does fall appreciably would reflect too favourably on the conditions of manufacture.

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315. THE EFFECTS OF COMMERCIAL PROCESSING AND OF STORAGE ON SOME NUTRITIVE PROPERTIES OF MILK. COMPARISON OF FULL-CREAM SWEETENED CONDENSED MILK AND OF EVAPORATED MILK WITH THE ORIGINAL RAW MILK

By KATHLEEN M. HENRY, J. HOUSTON, S. K. KON and S. Y. THOMPSON,
The National Institute for Research in Dairying, University of Reading

The present work is a continuation of our earlier studies(1, 2, 3, 4) on the effect of various commercial heat treatments on the nutritive value of milk. It deals mainly with sweetened condensed milk, a widely used and important product; the opportunity was also taken to add to information previously obtained(3) for unsweetened condensed (evaporated) milk. Through the kindness of Messrs Aplin and Barrett, Ltd., Frome, samples of raw milk and samples of full-cream sweetened condensed and of evaporated milk prepared from it were obtained on nine separate occasions. Condensed milk prepared on one of these occasions was stored and the effects of storage on its nutritive properties were studied. For convenience full-cream sweetened condensed milk will be called condensed milk and full-cream unsweetened condensed milk, evaporated milk.

EXPERIMENTAL

The preparation of the milks

The processing was carried out twice a week between 8 August and 5 September 1940. On 8 and 12 August the full-cream sweetened condensed milk was obtained from a full-scale commercial batch; otherwise the milks were made in small-scale experimental plants. This was unavoidable, as the two types of processed milk studied were not being manufactured simultaneously by Messrs Aplin and Barrett, and yet it was essential to have them made at the same time from a common bulk of raw milk.

(1) *Raw milk*

The source and treatment were as already described(3). Certain tests (vide infra) were carried out at once in the factory. For other tests samples of the milk were rapidly cooled, under gentle stirring, to 35° F. in an ice-cream mixer, placed in cooled Thermos flasks and either brought to Reading on the same day for chemical tests or sent by passenger train.

(2) *Full-cream sweetened condensed milk (condensed milk)*

(*a*) *Commercial method.* 900 gal. of new milk were run over an open tinned-copper forewarmer which raised the temperature from 45 to 100° F. and then flash-heated in a stainless steel internal tubular heater to 180° F. and held at that temperature for approximately 20 min. The milk was held in 400 gal. tinned-copper tanks in which a small amount of naked copper was exposed and during holding sugar was added as a 65%

solution raised to the boiling-point. The milk was concentrated in a tinned-copper batch evaporator, from which some tin was missing, for approximately 3 hr. at 115–120° F. Immediately on completion of evaporation the milk was run into tinned-copper cooling vats, water-jacketed and containing rotating water-cooled coils. In these the milk was gradually cooled to 65° F. in the course of $3\frac{1}{2}$ hr., and was then filled into 6 oz. cream tins.

(*b*) *Small-scale procedure.* 10 gal. of new milk were heated to 175° F. by passing through a stainless steel tubular heater and the requisite amount of sugar was dissolved in it. The liquid was further heated by steam injection to 180° F. and held at this temperature for 15 min., after which it was condensed in the course of approximately 45 min. in a small tinned-copper batch evaporator at 120° F. After condensing, the milk was allowed to cool to 95° F. and was then filled into 6 oz. cream tins without further cooling and without agitation. No facilities were available for controlled cooling of such small quantities, and consequently the lactose crystals were abnormally large.

(3) *Full-cream unsweetened condensed milk (evaporated milk)*

To 10 gal. of new milk 4 g. of sodium citrate were added and the liquid heated by steam injection to a temperature of 203° F. and held at this for 15 min. The milk was evaporated at 120° F. in about 35 min. in a small tinned-copper batch evaporator and was then immediately homogenized in a single-stage 'Brush' homogenizer working at a pressure of 1500 lb./sq. in. After filling into 4 or 6 oz. cream tins it was sterilized by bringing the temperature to 235° F. in the course of 15 min. and holding at this temperature for a further 15 min. The tins were then rapidly cooled to 90° F.

Assay of the milks

The biological value and digestibility of the proteins (nitrogen) of the milks were measured on rats. Vitamin A and carotene, vitamin B_1, riboflavin and vitamin C were measured by chemical or physical methods. These latter tests were done on four occasions, on 8, 15, 22 and 29 August 1940.

Methods of estimation of vitamins

A. *Raw milk*

Vitamin C. On each occasion the reduced form of ascorbic acid was measured at the factory immediately *before* processing by the method of Kon & Watson (5). The proteins were precipitated as described by Knight, Dutcher & Guerrant (6). The milk serum was then saturated with H_2S and the total ascorbic acid measured on the following day at Reading (5).

Vitamin B_1. Milk was placed in sterile Thermos flasks with a few drops of xylene to check bacterial growth, and brought back to Reading. Both the free and total vitamin B_1 content was measured by the method of Houston, Kon & Thompson (7).

Riboflavin. 20 ml. of milk and 20 ml. of redistilled methanol were heated at the factory for 20 min. to 60° C. in a brown glass bottle. After cooling the mixture was brought back to Reading and the riboflavin measured fluorimetrically (3).

Vitamin A and carotene. Milk was put into sterile Thermos flasks at Frome and brought back to Reading where vitamin A and carotene were measured by the method of Henry, Kon, Gillam & White (8). The Lovibond Tintometer used was standardized against a vitamin A concentrate of known potency and pure β carotene.

Fat and total solids. Fat was measured by the Gerber method and the total solids according to Golding (9).

B. *Processed milks*

Immediately after manufacture the processed milks were packed in 4 or 6 oz. tins and brought back to Reading where they were analysed on the following day. For this purpose they were diluted with distilled water so that 40 g. (occasionally 30 or 50 g.) of milk were contained in 100 ml. of the solution. Vitamin A and carotene, vitamin B_1, riboflavin and ascorbic acid were then measured as already described for the original raw milks.

EFFECTS OF PROCESSING

Biological tests

Comparison of the biological value and digestibility of the proteins of condensed, evaporated and raw milk

Experimental. The three milks were assayed by the method of Mitchell (10, 11). Four groups of three litter-mate female rats, weighing 50–65 g. when 25–26 days old were placed in glass and stainless steel metabolic cages. The experimental technique was as described in detail by Henry, Kon & Watson (12).

Fresh samples of the three milks were received twice a week, the raw milk was placed in the ice chest and a fresh Thermos flask of raw milk and fresh tins of the evaporated and condensed milks were opened daily. The evaporated and condensed milks were reconstituted with distilled water so that they had the same fat content as the original raw milk. Daily determinations of fat and total solids were made on the three milks as fed. Each fresh batch of milks was analysed for nitrogen, Table 1 gives the analyses of the reconstituted milks for nitrogen, fat and total solids, it also gives the analysis of the nitrogen-free diet.

Table 1. *Analyses of the raw milk and of reconstituted evaporated and condensed milks used in the nitrogen metabolism experiment*

	Raw milk			Evaporated milk			Condensed milk		
Date	% fat	% total solids	g. N/100 ml.	% fat	% total solids	g. N/100 ml.	% fat	% total solids	g. N/100 ml.
12. viii. 40	3·54	12·21	0·510	3·56	12·35	0·524	3·92	28·68	0·552
15. viii. 40	3·47	12·09	0·539	3·40	12·33	0·528	3·50	27·40	0·544
22. viii. 40	3·45	12·08	0·472	3·64	12·48	0·483	3·45	28·63	0·544
26. viii. 40	3·53	12·24	0·509	3·55	12·51	0·520	3·85	27·55	0·547
29. viii. 40	3·43	11·92	0·481	3·80	13·01	0·537	3·90	27·90	0·543
2. ix. 40	3·55	12·19	0·495	3·57	12·29	0·504	3·89	27·19	0·538
5. ix. 40	3·41	11·99	0·473	3·46	11·95	0·488	3·70	27·02	0·509

Analysis of N-free diet: 0·0363% N, 8·81% moisture.

During the milk feeding periods the milks and the nitrogen-free basal diets were fed in proportions estimated to yield a mixture containing about 1·2% N on the dry basis. The undiluted condensed milk contained about 43% sugar and therefore this milk supplied more calories than the other milks; due allowance was made for this by feeding a correspondingly smaller amount of the basal diet. At the end of the experiment it was found that on the basis of total solid intake, the raw milk diet contained on the average 1·08% N and the evaporated and condensed milk diets 1·11 and 1·12% N respectively. This corresponds to protein (N × 6·38) levels of 6·9, 7·1 and 7·1 respectively.

The diet and milks were fed separately during the first 3 days of the first experimental period, i.e. before collection of urine and faeces was started, the diet being fed mixed with half its weight of water. Several rats left considerable amounts of their milk during this period. It was then decided to adopt a method of feeding previously used in this laboratory (12) in which milk instead of water was mixed with the diet, the balance of the milk being fed separately. The animals were not given water to drink during the milk feeding periods as they tended to leave some of their milk when water was available. Distilled water was given to drink during the first and last periods when the low egg-nitrogen diet was fed. All animals received each milk in turn during the course of the experiment. Food intakes were not equalized between litter-mates, as by this method of feeding each animal served as its own control.

Results. Table 2 gives the individual nitrogen metabolism data, Table 3 the mean figures for the biological value and the true digestibility of the proteins of the three milks, Table 4 the statistical analysis of the results. 'Student's' *t*-test as described by White (14) was applied. When comparing two milks the results obtained on the same rat in different periods were used. The results for rat no. 11 are omitted from the averages as this animal consistently refused to consume all the evaporated milk it was offered.

Table 3 shows that the biological values of the proteins of the three milks lie close together and Table 4 that the small differences in favour of raw milk are without statistical significance. On the other hand, the true digestibility of the condensed milk was definitely superior to that of the raw and evaporated samples. The difference between the latter two was without statistical significance.

In a previous experiment (3) we obtained figures of 80·44 and 90·87 respectively for the biological value and true digestibility of the proteins of evaporated milk. The milk had then been fed entirely mixed with the diet and the experiment had suggested that evaporation is more injurious to the proteins of milk than spray-drying. There is nothing, however, in the present experiment to show that evaporation exerted any untoward effects on the proteins of milk; the higher digestibility of condensed milk is an expression not of a deterioration in the course of evaporation but of an improvement due to the condensing process. It should be remembered that there is evidence that for children the nitrogen of evaporated milk is more available than that of liquid milk (15, 16).

The biological value of 85·6 now obtained for raw milk may be compared with the value of 90 reported by Fairbanks & Mitchell (17) for liquid skim milk at an 8% level of protein intake.

Chemical and physical tests

The results of the tests described on p. 330 are given in Table 5 where values for the original raw milks and for the corresponding condensed and evaporated products will be found. In order to assess the effects of the processing on the various vitamins contained in raw milk, the degree of concentration in manufacture was calculated from the measured total milk solid contents of the original milk and of the two heat-treated products. Due allowance was made for cane sugar added to the extent of 43% to the sweetened condensed milk. Values for the vitamin content of the condensed and evaporated milks divided by the appropriate concentration factors are given at the bottom of Table 5, while the changes due to processing calculated from these figures are quoted in Table 6.

Rat no.

1 2 3 4 5 6 7 8 9 10 11 12

1 2 3 4 5 6 7 8 9 10 11 12

1 2 3 4 5 6 7 8 9 10 11 12

1 2 3 4 5 6 7 8 9 10 11 12

1 2 3 4 5 6 7 8 9 10 11 12

Table 2. *Individual nitrogen metabolism data*

Rat no.	Milk tested*	Weight of rat (g.) Initial	Final	Solid intake (g.) (dry basis) Diet	Milk	N intake mg.	Faecal N mg.	Urinary N mg.	Biological value	True digestibility
				Low egg-nitrogen diet						
1	—	64	72	41·67	—	—	93·2	83·9	—	—
2	—	63	71	42·95	—	—	90·5	88·6	—	—
3	—	62	69	39·56	—	—	80·6	76·4	—	—
4	—	53	59	35·53	—	—	81·5	64·8	—	—
5	—	52	54	31·02	—	—	82·8	69·1	—	—
6	—	53	57	32·15	—	—	80·1	67·1	—	—
7	—	50	56	33·74	—	—	85·8	59·1	—	—
8	—	51	54	30·68	—	—	82·0	67·1	—	—
9	—	52	57	34·23	—	—	77·8	66·6	—	—
10	—	59	63	36·12	—	—	94·9	71·3	—	—
11	—	58	65	38·38	—	—	91·1	70·3	—	—
12	—	58	62	36·08	—	—	88·2	71·6	—	—
				First experimental period						
1	R	73	74	21·17	7·09	305·8	74·3	130·4	84·4	97·0
2	E	71	75	21·54	8·00	340·2	99·0	129·1	89·2	91·1
3	C	68	71	11·04	17·13	314·7	72·2	150·5	74·7	95·6
4	R	62	64	21·86	7·78	335·9	106·0	98·4	89·3	88·7
5	E	57	61	19·48	6·76	287·6	78·6	123·2	81·0	97·1
6	C	62	72	14·38	24·13	442·6	90·7	129·7	87·6	100·0
7	R	55	59	20·05	6·95	300·1	80·0	95·7	88·0	96·7
8	E	58	58	17·77	5·03	315·4	66·8	101·2	89·1	98·0
9	C	61	64	11·24	16·19	298·6	73·4	121·8	82·0	99·1
10	R	59	63	18·11	6·00	260·3	68·9	119·0	79·0	98·3
11	E	60	60	17·43	4·44	190·9	77·1	127·8	61·2	98·4
12	C	61	71	14·69	24·17	444·0	94·0	145·3	83·5	100·0
				Second experimental period						
1	C	78	90	17·25	28·36	513·4	98·3	179·5	82·0	100·0
2	R	82	94	35·52	13·00	518·4	137·3	136·2	95·6	97·4
3	E	71	83	27·67	10·29	408·4	111·1	137·2	83·6	92·3
4	C	72	82	16·37	27·20	491·5	105·9	150·5	84·2	98·8
5	R	67	77	29·36	10·66	425·3	112·4	132·9	86·7	98·7
6	E	76	87	32·46	12·22	484·3	117·0	125·4	91·0	98·3
7	C	64	80	17·11	28·92	523·5	114·7	118·7	91·4	100·0
8	R	65	75	31·10	11·48	457·3	140·8	113·7	90·8	93·7
9	E	67	74	25·64	9·27	367·8	118·6	125·9	83·5	90·6
10	C	66	82	17·48	28·98	524·8	99·6	120·9	90·6	100·0
11	R	66	78	30·42	10·86	433·0	110·6	117·7	88·6	98·6
12	E	71	80	26·92	10·36	411·0	127·5	118·7	88·6	92·3
				Third experimental period						
1	E	98	107	34·94	12·95	529·6	157·5	172·2	84·5	92·1
2	C	103	108	17·75	26·50	482·5	110·3	192·6	88·3	100·0
3	R	91	104	36·98	13·16	527·2	153·2	171·0	80·5	91·2
4	E	88	97	30·02	11·23	460·5	132·6	168·0	79·0	91·6
5	C	85	98	19·51	31·85	578·9	115·7	168·7	86·1	100·0
6	R	96	103	40·38	14·72	589·8	165·9	185·8	82·8	94·4
7	E	84	86	26·30	8·25	339·2	114·8	154·0	77·6	93·5
8	C	82	92	19·46	31·58	573·9	172·9	190·6	80·0	93·1
9	R	82	92	31·52	11·03	442·3	128·0	145·3	83·9	94·9
10	E	86	96	32·86	11·88	486·4	160·1	176·4	78·3	92·6
11	C	86	101	20·31	31·88	579·8	125·9	201·1	79·5	100·0
12	R	88	94	30·66	11·35	454·9	177·0	157·2	80·5	85·5
				Low egg-nitrogen diet						
1	—	108	115	56·89	—	—	140·4	94·2	—	—
2	—	102	97	30·59	—	—	90·9	126·9	—	—
3	—	108	117	41·46	—	—	89·7	90·7	—	—
4	—	99	115	48·18	—	—	109·6	80·7	—	—
5	—	100	105	43·22	—	—	115·8	86·6	—	—
6	—	108	121	53·56	—	—	128·1	91·2	—	—
7	—	90	94	44·60	—	—	121·2	85·7	—	—
8	—	94	99	50·74	—	—	131·2	83·5	—	—
9	—	95	104	55·62	—	—	141·6	78·6	—	—
10	—	96	108	45·32	—	—	127·5	77·9	—	—
11	—	103	119	52·23	—	—	139·7	87·0	—	—
12	—	96	101	47·69	—	—	128·5	78·7	—	—

* R = Raw, E = Evaporated, C = Condensed.

(1) *Condensed milk.* Owing to the presence of sugar which effectively inhibits bacterial growth the heat treatment applied to this product is much less drastic than that involved in the preparation of evaporated milk. One would therefore expect less destruction of vitamins, and this is borne out by our findings. It will be seen in the first instance that neither carotene nor vitamin A have been affected. The small apparent loss of carotene just as the apparent small gain in vitamin A are well within the limits of experimental error. Riboflavin is also unchanged, and these results tally with the relative heat stability of these three factors.

Some destruction of vitamin C has taken place, the loss amounting to 10% of the total vitamin C and to 27% of that present originally in the reduced form. The latter is more stable than dehydroascorbic acid and its apparent greater loss is probably explained by reversible oxidation proceeding simultaneously with destruction of the oxidized form.

Table 3. *Mean biological values and true digestibilities of the proteins of the different milks*

Experimental period	Biological value			True digestibility		
	Raw milk	Evaporated milk	Condensed milk	Raw milk	Evaporated milk	Condensed milk
I	85·18	86·43	81·95	95·18	95·40	98·68
II	92·47	85·60	87·00	96·60	93·38	99·70
III	81·93	79·85	84·80	91·50	92·45	97·70
Mean	85·59	84·13	84·58	94·23	93·74	98·78

Table 4. *Statistical analysis of the data of Table 3*

Difference tested	Biological value			True digestibility		
	Raw-evaporated	Raw-condensed	Evaporated-condensed	Raw-evaporated	Raw-condensed	Evaporated-condensed
Difference	+1·46	+1·01	−0·45	+0·64	−4·55	−5·19
S.E.M.	±1·93	±1·92	±2·34	±1·39	±1·29	±1·25
*P**	1 : 2	1 : 2	1 : 1	1 : 2	1 : 185	1 : 500
	Not significant	Not significant	Not significant	Not significant	Significant	Significant

* *P* = Probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

The final effect is very mild and the loss in antiscorbutic power is less than in any other heat treatment of milk so far studied by us (1, 2, 3, 4).

It is of interest to recall that the value of sweetened condensed milk as a source of vitamin C was noted by Hume (18) as early as 1921.

The destruction of vitamin B_1 is even less and the net decrease of 3·5% is probably just on the borderline of significance. As freshly prepared, condensed milk thus retains to a remarkable degree the nutritive properties of the original raw product.

(2) *Evaporated milk.* Here again, though the heat treatment was much more severe, vitamin A and carotene have survived undamaged. This confirms our earlier findings (3). Mean figures indicate a slight, 5%, loss in riboflavin. Individual measurements fluctuate from an apparent gain of 1·2 to a loss of 11·5% and it is a matter of speculation whether the over-all figure should be accepted as real. The loss, if any, in riboflavin is negligible and this finding agrees with the tentative suggestion regarding its stability put forward by us in an earlier publication (3).

Table 5. *The vitamin content of raw milk and of condensed and evaporated milks prepared from it. Results of four separate measurements*

		Values per 100 g. product										
		Vitamin C mg.		Vitamin B_1 μg.			Carotene μg.					
Type of milk	Exp. no.	R.	T.	F.	T.	Riboflavin μg.	M. fat	N.S.R.	Vitamin A i.u.	Fat g.	Total milk solids g.	Concentration factor
Raw	1	1·50	1·85	23·5	36·5	137·6	29·4	28·4	65·0	3·30	11·96	—
	2	1·47	2·21	22·2	34·9	150·5	—	32·4	86·3	3·45	12·08	—
	3	1·87	2·59	19·9	35·8	146·2	29·1	30·2	78·4	3·35	11·98	—
	4	1·59	2·12	19·7	36·9	126·2	—	27·9	75·1	3·40	12·03	—
Mean		1·61	2·19	21·3	36·0	140·1		29·7	76·2	3·38	12·01	—
Condensed	1	2·38	4·84	60·0	96·2	392·8	83·0	80·1	201·3	9·54	34·59	2·89
	2	3·28	5·65	59·5	103·0	463·0	—	92·4	242·9	10·38	36·35	3·01
	3	4·75	6·90	60·5	99·5	421·0	84·3	84·3	224·2	9·58	34·25	2·86
	4	3·28	5·60	68·0	106·0	390·0	—	81·3	233·0	9·79	34·69	2·88
Mean		3·42	5·75	62·0	101·2	416·7		84·5	225·4	9·82	34·97	2·91
Evaporated	1	0·50	3·18	52·0	85·3	378·8	93·4	89·3	213·4	10·26	37·16	3·11
	2	1·00	3·32	47·7	75·0	428·3	—	99·8	268·3	10·73	37·54	3·11
	3	0·67	1·65	44·3	74·3	380·0	74·9	74·0	201·5	8·61	30·74	2·57
	4	0·53	2·40	49·7	77·7	395·3	—	91·8	249·9	10·68	37·74	3·14
Mean		0·68	2·64	48·4	78·1	395·6		88·7	233·3	10·07	35·80	2·98
Condensed, raw milk equivalent	1	0·82	1·67	20·8	33·3	135·9	28·7	27·7	69·6			
	2	1·09	1·88	19·8	34·2	153·8	—	30·7	80·7			
	3	1·66	2·41	21·2	34·8	147·2	29·5	29·5	78·4			
	4	1·14	1·94	23·6	36·8	135·4	—	28·2	80·9			
Mean		1·18	1·98	21·4	34·8	143·0		29·0	77·4			
Evaporated, raw milk equivalent	1	0·16	1·02	16·7	27·4	121·8	30·0	28·7	68·6			
	2	0·32	1·07	15·3	24·1	137·7	—	32·1	86·2			
	3	0·26	0·64	17·2	28·9	147·9	29·1	28·8	78·4			
	4	0·17	0·76	15·8	24·7	125·9	—	29·2	79·6			
Mean		0·23	0·87	16·3	26·3	133·3		29·7	78·2			

R. = reduced; T. = total; F. = free; M. fat = measured in the fat; N.S.R. = measured in the non-saponifiable residue.

Table 6. *Vitamin losses caused by condensing and evaporating expressed as percentages of original values*

Exp. no.	Vitamin C		Vitamin B_1			Carotene		
	R.	T.	F.	T.	Riboflavin	Fat	N.S.R.	Vitamin A
				Condensed milk				
1	45·3	9·7	11·5	8·8	1·2	2·4	2·5	−7·1
2	25·9	14·9	10·8	2·0	−2·2	—	5·2	6·4
3	11·2	6·9	−6·5	2·8	−0·7	−1·4	2·0	0·0
4	28·3	8·5	−19·8	0·3	−7·3	—	−1·1	−7·7
Mean	26·7	10·0	−1·0	3·5	−2·3		2·2	−2·1
				Evaporated milk				
1	89·3	44·9	28·9	24·9	11·5	−2·1	−1·1	−5·5
2	78·2	51·6	31·1	30·9	8·5	—	0·9	0·0
3	86·1	75·3	13·6	19·3	−1·2	0·0	4·3	0·0
4	89·3	64·2	19·8	33·1	0·2	—	−4·7	−6·0
Mean	85·7	59·0	23·4	27·1	4·8		−0·2	−2·9

Abbreviations as in Table 5.

The losses incurred by the two more labile vitamins, B_1 and C, are, as would be expected, much more marked than for the sweetened product.

The net loss of vitamin C, 60%, is higher than that previously found by us(3). Woessner, Elvehjem & Schuette(19) have reported large variations in the ascorbic acid content of evaporated milk of commerce. Their findings differ from ours in that they found in the numerous samples examined hardly any dehydroascorbic acid, the bulk of the vitamin being present in the reduced form. In our samples, on the contrary, the reversibly oxidized form predominates. Again, as for condensed milk the apparent loss of the reduced form is greater and the explanation is doubtless the same. We are unable for the present to account for the difference between our observations and those of Woessner *et al.*(19).

The loss during evaporation of 27% of the total vitamin B_1 observed by us now is lower than that, about 50%, indicated by the biological tests of Henry, Houston, Kon & Osborne(3) with freshly evaporated milk but is in good agreement with the findings of Schlutz & Knott(20) and of Knott(21) who also examined the product shortly after manufacture. The former authors observed losses of from 20 to 34% and the latter from 23 to 35%. On the other hand, Daniels(22) has reported destructions ranging from 39 to 54% but her comparisons were between raw and processed milks of different origin.

Our findings show that as an end result more of the combined than of the free form of vitamin B_1 has been lost in the course of evaporation. This tallies with our earlier observations(4) for other types of processing, though the difference at present is less marked. This greater lability of the combined form may well only be apparent and due to the splitting off of free vitamin B_1 during processing.

Effects of storage on the vitamin content of condensed milk

Through the kindness of Dr C. H. Lea, tins of condensed milk were stored at the Low Temperature Research Station, Cambridge. No storage experiments were done with evaporated milk. Two temperatures were used: 15° C. to approximate to normal conditions of storage in this country, and 37° C. for accelerated tests, and also to give some indication of effects which might be expected in extreme tropical conditions.

The tins stored were those prepared in the third experiment (cf. p. 330 and Tables 5 and 6), i.e. on 22 August 1940. They were stored at Frome at 36° F. until 5 September (14 days) and then dispatched to Cambridge. Owing to difficulties in transport the milks did not arrive in Cambridge until 14 September 1940. If a temperature coefficient of 2·2 per 10° C. is assumed, the time elapsing between the manufacture of the milks and their receipt at Cambridge would correspond to approximately 12 days' storage at 15° C. or 2–3 days' storage at 37° C. Samples were sent to Reading before storage commenced in order to find out if any deterioration had occurred. Thereafter they were taken from storage and dispatched to Reading for assay at times indicated in Table 7. The results of these tests are given in Table 7. It will be seen that there was no loss of vitamin A, carotene or riboflavin between manufacture and arrival in Cambridge. Some slight loss, however, possibly occurred in vitamin B_1 and vitamin C.

After 1 month's storage at 37° C. the condensed milk showed a brown discoloration which progressively increased on further keeping until after 6 months the milks were toffee coloured. An unpleasant smell also developed. After 3 months' storage small

Table 7. *Changes in the vitamin content of condensed milk during storage at 15 and 37° C. Values for 100 g. of product*

Values in brackets indicate percentage destruction.

	Vitamin C mg.				Vitamin B_1 µg.				Riboflavin µg.		Carotene µg.				Vitamin A i.u.	
	15° C.		37° C.		15° C.		37° C.				15° C.		37° C.			
Time of storage	R.	T.	R.	T.	F.	T.	F.	T.	15° C.	37° C.	M. fat	N.S.R.	M. fat	N.S.R.	15° C.	37° C.
Freshly prepared	4·75	6·90	4·75	6·90	60·5	99·5	60·5	99·5	421·0	421·0	84·3	84·3	84·3	84·3	224·2	224·2
At start of storage*	4·52	5·70	4·52	5·70	60·8	95·0	60·8	95·0	415·0	415·0	80·5	83·3	80·5	83·3	239·5	239·5
2 weeks	—	—	4·08	6·43	—	—	63·5	85·3	—	428·0	—	—	80·5	83·3	—	239·5
4 weeks	3·83	5·74	3·39	5·47	58·3	88·0	63·5	82·7	425·0	428·0	78·6	78·6	76·6	78·6	247·2	247·2
6 weeks	—	—	2·98	4·43	—	—	40·4	48·0	—	415·0	—	—	79·5	79·5	—	243·3
2 months	3·74	6·38	2·81 (41)	4·27 (38)	54·2	81·3	36·3 (40)	43·3 (57)	410·0	432·0 (0)	94·8	95·8	95·8 (0)	92·9 (0)	231·8	231·8 (0)
3 months	3·46 (27)	5·88 (15)	2·15	3·40	54·2 (10)	81·4 (18)	19·3	23·6	427·0 (0)	403·0	83·3 (0)	90·1 (0)	88·1	89·1	231·8 (0)	218·4
4 months	—	—	2·51 (47)	3·13 (55)	—	—	17·5 (71)	16·2 (84)	—	383·0 (9)	—	—	89·1 (0)	89·1 (0)	—	247·2 (0)
5 months	—	—	1·89	3·02	—	—	8·3	18·4	—	293·0	—	—	88·1	89·1	—	224·2
6 months	3·44 (28)	5·33 (23)	1·60 (66)	2·52 (64)	52·5 (13)	79·3 (20)	3·1 (95)	6·8 (93)	415·0 (0)	275·0 (35)	81·4 (0)	83·3 (0)	81·4 (3)	76·6 (9)	224·2 (0)	224·2 (0)
9 months	3·27	5·19	—	—	51·0	74·5	—	—	426·0	—	86·2	84·3	—	—	218·4	—
1 year	3·15 (34)	3·99 (42)	—	—	50·5 (17)	71·0 (29)	—	—	433·0 (0)	—	98·7 (0)	93·9 (0)	—	—	224·2 (0)	—

* Time taken in transit was roughly equivalent to 12 days at 15° C. and to 2–3 days at 37° C.

Abbreviations as in Table 5.

mould colonies ('buttons') were observed in most tins. As time went on the 'buttons' grew in size and the largest observed was almost $\frac{3}{4}$ in. in diameter. These 'buttons' were rejected before the milk was sampled.

Milks held at 37° C. became gelatinous making reconstitution increasingly difficult.

It should be noted that the condensed milk was prepared in a small experimental plant. As would be expected under the circumstances sugar separated out from the condensed milk but these crystals were not very large.

No brown colour developed at 15° C. After 12 months' storage the milks looked normal in appearance but had an unpleasant smell. No 'buttons' were found at this temperature.

Results

The results are given in Table 7. These show that there was no loss of vitamin A or carotene during storage for 6 months at 37° C. or storage for 12 months at 15° C.

There was a marked decline in all the water-soluble vitamins with the exception of riboflavin in the milks stored at 15° C. and an even greater loss at 37° C. The percentage losses are given in Table 7.

Thus the vitamin C activity declined at 15° C. by 15% after 3 months, 23% after 6 months and 42% at the end of a year. The losses of vitamin B_1 were also quite appreciable, 18, 20 and 29% respectively. At 37° C. nearly 40% of the vitamin C was lost in 2 months and the destruction increased to 55 and 64% respectively after 4 and 6 months of storage. At this temperature vitamin B_1 proved more labile than vitamin C and 57% was lost after 2 months and 84 after 4 months. The destruction after 6 months was almost complete and less than one-tenth of the vitamin originally present survived this length of storage.

It is of interest that marked losses of vitamin B_1 in the course of storage of evaporated milk have been noticed by Knott(21) who is of the opinion that the destruction is due to simple cleavage of the vitamin B_1 molecule into the pyrimidine and thiazole components.

Storage at 15° C. for a year had no effect on the riboflavin content of condensed milk but in the accelerated test some evidence of destruction could be noticed after 4 months, and after 6 months about one-third of the original riboflavin had been lost.

SUMMARY

1. Full-cream unsweetened condensed milk (evaporated milk) and full-cream sweetened condensed milk (condensed milk) were prepared simultaneously on several occasions each time from a common bulk of raw milk. Heat treatment in the preparation of evaporated milk is, of course, much more severe than with condensed milk.

(*a*) There was no loss of vitamin A, carotene or riboflavin in either of the processes.

(*b*) The mean loss of vitamin C as compared with the raw milk was 10% for condensed and 60% for evaporated milk.

(*c*) The vitamin B_1 losses were 3·5 and 27% respectively.

(*d*) Tests on rats showed that the biological values and true digestibilities of the proteins of the raw, condensed and evaporated milks were respectively 85·6 and 94·2, 84·6 and 98·8, 84·1 and 93·7. The differences in biological value are without statistical

significance but the digestibility of the condensed milk was significantly higher than that of the other two milks.

2. The condensed milk was stored for 1 year at 15° C. and samples were periodically examined for vitamin content.

(*a*) There was no loss during that period of either vitamin A, carotene or riboflavin.

(*b*) About 30% of the vitamin B_1 originally present was lost.

(*c*) The vitamin C loss amounted to 42%.

(*d*) Much larger losses were noticed during accelerated storage tests at 37° C. Thus after 6 months 64% of vitamin C, 93% of vitamin B_1 and 35% of riboflavin were destroyed. Vitamin A and carotene remained unchanged.

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REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION E. DISEASES OF DAIRY CATTLE

I. Mastitis
II. Contagious abortion
III. Tuberculosis

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I. MASTITIS

The available literature during the period under review (March 1940–March 1942) is chiefly British and American. In Britain the main emphasis has tended to change from the detection and isolation of all positive cows, including latent carriers, to the detection of only those cows clinically affected and which can be identified by simple methods in the byre. This is mainly due to the advice given to the Ministry of Agriculture by the Agricultural Research Council and the National Veterinary Medical Association. Both bodies put forward recommendations for the control of mastitis and these are embodied in the recent National Scheme of Control (1). The memorandum on the scheme lists the principal factors predisposing to mastitis as (1) overstocking, (2) hurrying the cows before milking, causing damaged teats, (3) incompetent milking by inexpert workers, and (4) ill-managed milking machines. As bacteriological examination of the milk of all cows is not possible the measures of control suggested are (1) daily use of the strip cup, (2) periodic clinical examination, (3) segregation or milking last of the known infected cows, (4) milkers' hands to be scrubbed and washed in disinfectant before and after milking each animal, (5) sterilization of the milking machine and dipping the teat cups in disinfectant after each cow has been milked, and (6) udder cloths to be kept in disinfectant when not in use.

The treatment suggested is sulphanilamide *per os* in acute cases in milking cows and an injection of an acridine dye in chronic cases, especially when nearly or quite dry. Sulphanilamide is supplied at a reduced charge by the state, and free laboratory facilities are available to type the causal organism in cases of doubt.

No control measures or treatment are suggested for types of mastitis other than that caused by *Streptococcus agalactiae*, though it is noted that filling the teats with an antiseptic emulsion before turning out dry cows may be a useful preventive measure in cases due to *Corynebacterium pyogenes*. The scheme has only recently come into operation and it is too early to assess the results.

Similar methods of control have been described by Hucker (2), who reported that the percentage of cows condemned or segregated for mastitis fell from 2·2 to 1·7 in 1,491,325 cows examined over a period of 5 years. He emphasizes the value of proper hygiene, which includes the sterilization of the hands of the milkers and the teat cups of the machines. Harrison (3) has shown that the hands of the milkers habitually harbour *Str. agalactiae* even in herds where the mastitis infection rate as revealed by bacteriological examination is either low or nil. He has also shown that a detergent is necessary for the removal of the organism from the hands, and that soap and water are the most efficient agents. McCulloch (4) found that non-medicated hard soap was at least as good a disinfectant as hypochlorite at 100 p.p.m. available chlorine. Medicated soaps had no advantage over non-medicated. He advocates household lye, i.e. 97·5% NaOH, at a dilution of 1:400 for the byre floors and stalls. Hay (5) found that hypochlorite (50 p.p.m.) was not successful in sterilizing teat cups even if these were previously rinsed in water, and he therefore advocates the treatment of teat cups with live steam for 30 sec. between each cow, followed by rinsing in hypochlorite. Seelemann (6) also emphasizes the importance of hygienic measures and the need for proper co-operation by the farm personnel. He considers infection to be independent of age and milk yield but to be greatly influenced by the types of milking practised and the general health of the herd. That the method of milking is of fundamental importance was shown by Watts (7) who compared the figures from 44,000

tests of 2858 cows in 39 herds and showed that there was a statistically significant difference between the infection rates in herds according to the type of milking machine used. There was also a significant difference in the amount of clinical mastitis.

Where proper attention can be paid to hygienic precautions considerable improvement may be expected. Christiansen (8) reports that in 2 years a herd of 207 animals was changed from 135 infected and 72 uninfected cows to 66 infected and 154 uninfected. He states, however, that complete isolation was possible. That this is almost essential is emphasized by Little (9) who describes the conditions in four herds. Two have been free of infection (as regards *Str. agalactiae*) for 3 and 4 years respectively, in another no infection has been found during a period of 9 years, while in the fourth, where the infected cows—though milked last—had to be kept in the same byre, fresh cases have continually occurred in the free animals. This may be due to transmission of the disease by flies as well as by handling, since Sanders (10) has shown by experimental evidence that *Musca domestica* (the housefly) and also *Hippelates* species (the fruit flies) can spread contagious mastitis. Flies fed on infected milk and then either placed on the teat orifice or set free in a screened enclosure with non-infected cows caused mastitis.

Another factor of importance appears to be intercurrent infection. This has been particularly noted with foot-and-mouth disease, a disease which is essentially debilitating in character. In Christiansen's work (8) the clear herd, when affected with this disease, developed a high incidence of contagious mastitis, 73 out of the 129 animals becoming positive. Kastli (11) also considers that foot-and-mouth disease increases the mastitis infection rate: thus in 3344 cows the infection rate was 4% before and 17% after an outbreak of the disease.

That local as distinct from general infections can favour infection with *Str. agalactiae* is maintained by Pounden & Johnson (12), who showed that an udder coccus resembling *Staph. albus* can stimulate the growth of *Str. agalactiae* as judged by titratable acidity in Hotis tests. Pounden & Zehner (13) suggest that the clinical signs of mastitis are produced by such acidity occurring in the udder. They injected into the udder whey produced by the action of *Str. agalactiae* on sterile milk and produced an acute inflammatory reaction similar to acute mastitis. Klein & Kleckner (14) found that in six cows, exposed to infection by suckling calves previously suckled on infected animals, the only quarters to be infected were those previously affected with *Staph. aureus*.

In addition to the interest now being taken in predisposing factors much work is being done on the habitat of *Str. agalactiae*. Thus Harrison (15) swabbed the nostrils and throats of 74 cows (69 of which showed *Str. agalactiae* in the milk), the vaginas of 20 virgin heifers and 56 cows (15 of which showed milk infection) and took faecal samples from 24 infected cows. No group B streptococci were recovered. Negative results were also recorded by Francis (16) for 100 pairs of bovine tonsils removed within 12–48 hr. of slaughter and for vaginal swabs from 48 cows, though streptococci of Minett's group 2 and group 3 were isolated.

Klein & Kleckner (14) swabbed the mouths of calves between half an hour and 14 hr. after suckling infected cows and found *Str. agalactiae* up to 8 hr. after suckling. The sucking of each other's teats is considered by Schalm (17) to be a cause of infection in young heifers. He found that in an infected herd in which the calves were allowed to suck each other's teats 5·7% of the heifers were infected when they calved. In a similar herd where the milk was pasteurized and the calves muzzled, no heifers were infected on calving. The evidence cannot be regarded as conclusive as the conditions were not strictly comparable, but it is certainly suggestive. The infection of heifers prior to lactation is, moreover, confirmed by Bryan, Moore & Campbell (18) who examined udder material after slaughter. Two of six heifers which had never lactated were found to be infected. Mattick, Shattock & Jacob (19) report that 4·5% of heifers tested while still in isolation after their first calving were positive for *Str. agalactiae*, and that 21% became positive during their first lactation.

Slanetz & Naghski (20) could not demonstrate *Str. agalactiae* in the udder tissues of five cows which had been repeatedly examined by the ordinary laboratory tests and found negative. They consider this shows the reliability of cultural tests on ordinary milk samples. They identified 573 out of 680 strains of streptococci isolated from cows with clinical evidence of mastitis as *Str. agalactiae*, and they agree with the English workers that this organism is the main cause

of chronic mastitis. Eighty-three strains of *Str. uberis* (Minett group 3), fifteen of *Str. dysgalactiae* (Minett group 2) and nine of *Str. faecalis* were also identified, but the infection produced by them was sporadic and not communicable. In a herd of 144 cows Murphy (21) found eighty-five quarters infected with *Str. agalactiae*, the strains being identified by their serological (all were group B Lancefield) and biochemical reactions. All strains fermented dextrose and lactose but not mannitol, inulin, raffinose or sorbitol, and split sodium hippurate but not aesculin. Methylene blue was not reduced. They differed, however, on their actions on trehalose and salicin. The smaller group of eighteen strains all fermented salicin but not trehalose, while the larger group of sixty-seven strains all fermented trehalose though their effect on salicin varied. There was no relationship between the action on trehalose and haemolytic power or virulence. That some degree of true variability does occur with *Str. agalactiae* has been suggested by Sherman, Greisen & Niven (22) who studied many thousands of daughter colonies from pure cultures, particularly in regard to haemolytic properties and ability to ferment lactose and salicin. While most of the cultures were remarkably stable these authors were able to isolate non-haemolytic variants from haemolytic parent cultures, but not vice versa. Negative strains were also obtained from strains able to ferment lactose and/or salicin, and positive strains from cultures which were originally unable to ferment these carbohydrates. Rantz & Jewell (23), from a study of 1179 strains of haemolytic streptococci belonging to groups A–K (Lancefield) isolated from human patients, consider that the type of haemolysis in deep blood agar plates is a valuable guide to the probable group of the strain. Most of the strains were group A (1104), and all showed a sharply defined zone of complete haemolysis of about 1·5–3·5 mm. diameter after 24 hr. The haemolysis produced by group B strains was not complete until after 48 hr. incubation, when it was surrounded by a hazy zone of partial haemolysis (Brown's double zone). Groups C–K produced a wide zone of haemolysis complete for 3 mm. but incomplete beyond. This zone increased on further incubation. This evidence does not agree with that of Gunnison, Luxen, Cummings & Marshall (24), who report that only half their group B strains produced a double zone of haemolysis. They studied 241 cultures from human sources (156 group A, 13 group B, 9 group C, 6 group D, 1 group F, 28 group G, 3 group H and 25 not grouped), and 320 from animal sources, mainly milk (12 group A, 263 group B, 15 group C, 2 group D, 4 group E, 10 group G, 1 group H and 13 ungrouped), and found that the various groups could not be identified by any combination of biochemical tests, though for preliminary differentiation into subgroups fermentation tests with sorbitol, trehalose, salicin, lactose and raffinose and the reaction in sodium hippurate were of value. They consider that the precipitin test is the simplest and most valuable for general diagnostic work. Nottbohm (25) examined 170 strains of streptococci by this test, though he used the formamide method of preparation which he considers preferable as it uses less media and is more specific in his hands. Forty of these strains were group B, thirty-nine from milk and one from a human throat. All were non-pathogenic, as were twelve strains of group C from cows. On the other hand, two strains of group C from human sources, ten from strangles in horses and six from other sources were all pathogenic to mice.

Seelemann & Flint (26) state that group C strains including *Str. dysgalactiae* can be divided into four subgroups. They found that sera from rabbits immunized with *Str. dysgalactiae* (group 2 Minett) was type specific and failed to react with antigens from other strains. The sera prepared with other group C strains reacted, however, with *Str. dysgalactiae* antigen. They also describe the technique they consider best for the production of group B serum. This consists of daily doses of formol vaccine for 5 consecutive days each week for 3 weeks, the animals being bled 3 days after the last dose. A similar method was used by Plastridge, Banfield & Williams (27) who pooled antisera against eleven serological types of *Str. agalactiae* and *Str. uberis*. They also prepared a serum against a single strain of *Str. dysgalactiae*. They found that the best medium to use for the preparation of specifically agglutinable suspensions was beef infusion broth with 2% difco proteose peptone (great emphasis is laid on the type of peptone used) 0·04% dextrose and 1% K_2HPO_4, *p*H 7·4. Two modifications of this medium were used for those strains which produced granular suspensions. In one 1% inactivated ox serum was added and in the other 0·1% dextrose was substituted for the 0·4% dextrose of the original medium. Of 242 strains of *Str. agalactiae* satisfactory antigens were obtained from 179 in the

main medium and a further 63 in one or other of the two modifications. The respective figures for 34 strains of *Str. uberis* were 20 and 9 and for 14 strains of *Str. dysgalactiae*, 2 and 7.

Diagnosis

Edwards's blood-agar medium continues to be the basal medium for comparative studies and is still used in most control work. The value of sodium azide in this medium has been confirmed by Lichstein & Snyder (28), who compared the effects of incorporating 6% NaCl, 2% antisera, alcohol and sodium azide. The NaCl inhibited streptococci, the antisera were only inhibitory against homologous strains of Proteus, and the alcohol haemolysed the blood. Sodium azide, however, inhibited the other groups of organisms while allowing *Str. agalactiae* to develop. More delicate methods are advocated by Mattick *et al.* (19) who have shown that where such methods are used many more cows are found to harbour *Str. agalactiae*, though the infection is often light and transient.

Edwards's liquid selective medium has been adversely criticized by Stirling (29) who tested 958 samples, of which 164 were positive. Only 93 of these (57%) were positive with Edwards's liquid medium. When modified by reducing the concentrations of sodium azide and crystal violet to half the proportions recommended by Edwards, the percentage of positives rose to 73, but the number of false positives which needed plating was much greater. Little (30), however, considers this method to be a valuable one, and points out that extracts for Lancefield precipitin tests can be prepared direct from the medium. McKenzie (31) has described a similar medium in which the inhibitors are thallium acetate 1/1000 and crystal violet 0·00005%. No other results on large numbers of samples tested in this medium have so far been published.

Other cultural methods recently developed are:

(1) The addition of brom-cresol purple and 1/500 sodium azide to the milk samples, which are then incubated (Little (30)).

(2) The microscopic examination of films from incubated milk samples containing brom-cresol purple (Hardenburg (32); Cone & Grant (33)).

(3) The microscopic examination of a standardized amount of milk after incubation for 18 hr. The samples consist of equal amounts of milk from each quarter mixed to give a bulk sample of each cow (Kleckner (34)).

(4) Inoculation of milk into a medium containing lactose and chalk. The acid produced by the fermentation of the lactose dissolves the chalk (Davis (35)). The author claims that *Str. agalactiae* is the only organism in freshly taken milk samples capable of producing this effect.

The basis of the second and third methods is the microscopic examination of films of incubated milk. This is also advocated by Kleckner (36), who found that this method gave 2·9% false positives and 0·3% false negatives in a series of 5796 negative and 1067 positive samples. He found, however, that the number of false positives was related to the byre conditions: thus in a herd under particularly good conditions only 0·7% of false positives were found, whereas under less hygienic conditions nearly 5% of false positives were recorded. He considers that this method is also applicable to composite cow samples and is a useful diagnostic agent, though all positive samples should be plated to distinguish groups 1, 2 and 3 (Minett).

Murphy & Hanson (37) have reported favourably on Whiteside's test which they modified slightly. They mixed 5 drops of fore-milk with 1 drop of *N* NaOH on a glass plate placed against a dark background. The mixture was stirred for 20 sec. and examined with reflected light for the presence of a precipitate. They consider this test is a useful presumptive test.

Other methods of examining milk for mastitis continue to be investigated. Thus Palmer, Kakavas & Hay (38) found that 8% of the quarters examined were brom-thymol blue positive and bacteriologically negative, while 29% were bacteriologically positive and brom-thymol blue negative. Kunicki-Goldfinger (39) suggests a combination of chloride, catalase, and alizarol tests with leucocyte counts, followed by plating of any positives on blood agar. He found that the agreement of individual tests with the blood-agar plate method was 64% for the catalase, 60% for the leucocyte count (>500,000 per ml.) and 23% for the chloride content. Cone (40), on the other hand, does not consider that any arbitrary value can be selected for the chloride content or leucocyte count as distinguishing between the normal and mastitis quarters. However,

should previous records for the particular quarter be known, he considers that any sudden rise in chloride content and leucocyte count, even though these should not reach the level usually considered abnormal, is evidence of infection. Vanlandingham, Weakley, Moore & Henderson (41) also compared the chloride and lactose contents, and the casein number in normal and abnormal quarters. The number of cows used was small (40) and the investigation was confined to one herd, but numerous and thorough separate examinations were made. Mastitis was diagnosed by the finding of haemolytic streptococci and by changes in the other tests used. These tests were made bi-weekly on samples of fore-milk. Fourteen of the forty animals were considered either positive or highly suspicious. The quarters were divided into three groups:

Group 1 consisted of negative quarters in negative cows.

Group 2 were negative quarters in cows with one or more of the other quarters affected.

Group 3 were the positive quarters.

No difference was observed between groups 1 and 2. In group 3 the authors found that quarter samples were necessary to detect changes due to mastitis, and even these did not reveal early cases, for bacteriological examinations were positive before chemical changes were demonstrated. They found that the percentage of chloride varied in the normal quarters from 0·09 to 0·224, the percentage of lactose from 3·3 to 5·4, the chloride-lactose number from 1·62 to 6·79, and the casein number from 69·4 to 84·3. When, as they recommend, the result from any quarter is compared with the most normal quarter of the same animal, they consider that differences of 0·02% in the chloride, 0·36% in the lactose, 0·6% in the chloride-lactose number and 2·9% in the casein number indicate mastitis. They do not recommend comparing the results from one animal with those from another.

Another indirect test, the electrical conductivity test, has recently been advocated by McCulloch (42) following the earlier work of Koeppe (43) and Plücker & Steinruck (44). The apparatus is small, cheap and portable and is suitable for use in the byre. McCulloch points out that, as would be expected, the results of this test closely parallel those of the chloride test as already noted by Davies (45). The conductivity test is therefore no more accurate than the chloride test. Corrections have also to be made for animals in early or late lactation.

Mastitis also affects the ascorbic acid content of milk. Reineke, Garrison & Turner (46) found that in the early stages of infection the reduction in ascorbic acid content was 10% but that as the disease advanced it fell by 30–50%. They do not consider that this is due to direct action by streptococci, since oxidation of the acid was found by *in vitro* experiments to be retarded by these organisms.

A useful article on the leucocyte picture in infected milk, especially with regard to phagocytosis, has been published by Hadwen (47). The plates accompanying this article are specially valuable in showing the appearance of cells when stained by Hastings stain.

Treatment

Evidence has been brought forward by Harris & Miller (48) that, while temperature is probably a factor of great importance in the bacteriological action of sulphanilamide, phagocytosis is only of secondary importance. They inserted collodion sacs containing large numbers of haemolytic streptococci into the peritoneal cavities of rabbits, and injected the animals subcutaneously with sulphanilamide. There was no phagocytosis of the bacteria and often no precipitable protein within the collodion sacs; nevertheless the organisms were killed by the action of the drug. Woods (49) suggests that this action is due to the inhibition of the enzyme reaction by which bacteria utilize *p*-aminobenzoic acid, a substance of similar structure to sulphanilamide but an essential factor for the growth of many bacteria including streptococci. This substance when added to media containing sulphanilamide neutralizes the inhibitory action of the latter and Woods emphasizes the importance of the medium in which the action of sulphanilamide is tested. If this is too rich no inhibitory action may be noted even though the strain or organism tested is susceptible to sulphanilamide.

This theory is supported by Green & Bielschowsky (50) who agree that the anti-sulphanilamide factor is *p*-aminobenzoic acid. They also agree that the essential action of this group of drugs

is their ability to block the bacterial enzyme whose natural substrate is *p*-aminobenzoic acid. They suggest that the active principle is the NH_2⬡S radical and found that the most potent compound *in vitro* was 4:4-diaminodibenzenedisulphide.

McEwan, Pizer & Paterson (51) have studied the action of sulphanilamide E.O.S. and 4:4-diaminodiphenylsulphone on mastitis. The dose of E.O.S. was 4 oz. followed by 2 oz. every 12 hr. for 14 days and of sulphone 1 oz. followed by ½ oz. every 12 hr. for 7 days. Slight toxic effects occurred with E.O.S. but not with sulphone, both had good effects on the clinical conditions but neither effected a bacteriological cure.

Other records of treatment are mainly of injections into the udder. Schalm (52) has investigated the uses of trypaflavin (neutral acriflavine) and found that though a 1:4000 solution was too concentrated in aqueous solution, the concentration could be raised to 1:500 if the dye was dissolved in a hypertonic (20%) sugar solution. After injection practically all could be milked out. His method, based on Steck's (53) cistern infusion method, is as follows: 200 ml. of 1:500 trypaflavine in sugar solution are injected as a preliminary rinse and immediately milked out into a graduated container. 400 ml. more are then injected, left in for 5 min., and again milked out into the same container. A total of 600 ml. are removed or until no more fluid is obtained. By this method he claims that 69% of fifty-five infected quarters were cured by one injection and 93% by two. American workers have recently published results with two new udder injections. The first, Novoxil, is 5% colloidal silver oxide in oil and has the disadvantage that the injected quarters become hard and swollen, especially in the Ayrshire breed. This swelling usually subsides without permanent damage. Klein, Kleckner & Biltz (54) have reported most favourable results with this substance. They injected sixty-seven quarters, 19% of which were clinically affected, and claimed 88% bacteriological cures, including ten of the nineteen clinically affected. Their methods varied. From 5 to 20 ml. were injected from 1 to 10 times at intervals of 12, 24, 44 and 72 hr. Three were injected once when dry with good results. On the whole it appears that three to five injections of 10 ml. at 24 hr. intervals gave the best results. These are more favourable than the preliminary results of Weirether, Anderson, Johnson, Plastridge & Jungherr (55) who reported that the three quarters they treated were only cleared for 11–37 days. Little, Dubos, Hotchkiss, Bean & Miller (56) also tested Novoxil as a curative agent and cleared five of ten quarters. These authors also treated thirty-two quarters with Gramicidin, a substance bactericidal to gram-positive bacteria, obtained by Dubos (57) from an organism of the *Bacillus* group isolated from soil. Of these thirty-two quarters twenty-eight were freed from *Str. agalactiae*, fourteen by a single injection of the drug. The dosage varied, but 120 mg. was the average. Where several injections were necessary they were carried out at 24 hr. intervals. These results are, however, open to criticism. The previous history of the cows was not known nor was the derivation of the infection. Most of the animals had received preliminary dosing with sulphanilamide and many were also injected with Novoxil. The conditions of management were radically changed for all treated cows since they were taken off the machine and hand milked, while the protein ration was reduced. No controls were used, so that it is not possible to assess what proportion of the cures were due to the treatment and what proportion, if any, to the changed conditions under which the cows were kept. In another article on the same subject Little, Dubos & Hotchkiss (58) stated that twenty-one of thirty-two quarters had been cured with Gramicidin and ten of seventeen with Novoxil. Further evidence of the value of Gramicidin has also been published by Tripp & Lawrence (59), who found that a dose of 160 mg. injected from one to three times was successful in clearing fourteen out of fifteen quarters infected with *Str. agalactiae*.

Martin (60) treated 130 quarters in seventy-one cows all in the one herd with Tyrothricin (commercial Gramicidin). Diagnosis was by the incubated smear method, i.e. samples were incubated for 18 hr. and a smear made. If long-chained streptococci were present the quarter was classed as positive and was treated with 20 ml. of an oily emulsion containing 30 mg. of Tyrothricin. After massage the quarter was left till the next milking, when it was stripped out. The injection was repeated weekly till the quarter was clear. The reaction to the injection was slight and 117 quarters (90%) were cured, the average number of injections required being 2·3. A more extensive comparative trial of various methods has been reported by Schalm (61). For

the flavine injections he used a modified version of Steck's method of cistern infusion and Stableforth & Scorgies's method of udder infusion, while he varied his doses of Novoxil and Gramicidin. His results were 90% of sixty-one quarters cured with Entozon, 82% of eighty-nine quarters cured with Gramicidin, 73% of thirty-four quarters cured with Novoxil, 59% of 181 quarters cured with Steck's method and 48·7% of forty-one quarters cured with Stableforth & Scorgies's method. He found that the percentage of cures varied greatly from herd to herd, the lowest being 53 and the highest 95. He considers that the treatment of the dry cow with Gramicidin or Novoxil may be valuable and states that infections due to *Str. uberis* and *Str. dysgalactiae* also responded to treatment. Results with staphylococcal infections were negative.

A further method has been suggested by Sanders (62) who injected seventeen infected cows with various dilutions from 1:900 to 1:1500 of iodine in liquid paraffin. Infection had been present from 2 to 12 months and he reports that all were cleared.

Other types of mastitis

An examination of fifty-four samples of milk from acute cases of mastitis led Ferguson (63) to suggest that acute mastitis has no relation to chronic mastitis due to *Str. agalactiae* but follows an injury to the teat meatus and is due to a variety of other organisms. Thus in her series of fifty-four cases only one was due to *Str. agalactiae*, while in twenty-two a member of the coliform group was isolated, in eight a *Staphylococcus*, in ten a β-haemolytic *Streptococcus*, in four *Corynebacterium pyogenes*, and in others group 2 and group 3 streptococci. Even in 109 subacute cases of mastitis she considered the causal organism to be a *Staphylococcus* in forty-one cases, *C. pyogenes* in eleven, a *Streptococcus* in twenty-five and coliform organisms in three.

Little (64) also draws attention to the importance of group 2 and group 3 infections. He found that group 2 was more often acute than group 3, which was usually benign. Both types, but more especially group 2, led to chronic atrophy of the quarters affected. He notes that in many cases group 3 streptococci were only found on a single examination and had disappeared before the next routine sample was tested.

Another *Streptococcus* which has been reported as causing infection and damage in the udder is group G. Miller & Heishman (65) isolated this organism from twenty-eight animals in a herd of forty-four which was suffering considerable losses from clinical mastitis. Of the nineteen animals clinically affected thirteen were shedding group G streptococci. Another unusual *Streptococcus* was found by Palmer, Kakavas & Hay (66) in an investigation into the causes of mastitis in heifers. It reproduced the disease when injected into normal cows. The largest proportion of cases was, however, due to staphylococci and when this occurred in dry heifers the quarters often went blind or became fibrotic without showing any symptoms. Milking cows, however, usually recovered from staphylococcal infection. Shattock & Mattick (67) report that 92 out of 428 cows free from *Str. agalactiae* were infected with haemolytic staphylococci. In no case was there any clinical symptom of disease, though there was some change in the chemical composition and *p*H of the milk. They urge the use of plain blood-agar plates instead of Edwards's medium if a true picture of the extent of infection in a herd is required, since the latter inhibits the growth of staphylococci.

The fermentation of mannitol and the production of toxin, pigment, haemolysin and coagulose by staphylococci has been studied by Bell (68). The strains were all of animal origin and consisted of fifty-four *Staph. aureus* and seven *Staph. albus*. Only one of the latter was haemolytic. Toxin was produced by roughly half the *aureus* strains and correlated perfectly with the production of coagulose for human and equine plasma but not with the fermentation of mannitol since many non-toxin producing strains also fermented this substance. The toxin produced was of the alpha type, though some also produced beta toxin. None was found to be able to produce beta toxin only.

The production of alpha toxin by a haemolytic *Staphylococcus* is dependent on the relative concentration of oxygen and carbon dioxide, and this has been shown by Casman (69) to vary, in a closed system, with the total volume of gas. Casman found that by bubbling a mixture of these gases through a veal infusion broth with 2% Difco proteose peptone and 0·7% sodium acetate added, *p*H 6·8, the toxin produced by any given strain was increased though it did not

reach the strength of that obtained by the semi-solid agar method. When the medium was inoculated and exposed in shallow dishes to a mixture of 20% CO_2 in O_2 the toxin produced equalled that of the semi-solid agar method provided the dishes were gently rocked through an angle of 45° every 3 sec. Similar results are reported by Favorite & Hannon (70) using a medium composed of acid casein hydrolysate and glucose with vitamin B and nicotinic acid. After inoculation the medium is slowly rotated in the presence of CO_2 and the yield of toxin is said to equal that obtained by usual semi-solid agar technique.

Staphylococcus toxin can be detoxicated by peptic digestion at *p*H 4·2 and the toxoid so formed was found by Parfentjev, Clapp & Wald-Schmidt (71) to be highly antigenic and to cause less local reaction than formolized toxoid when injected intracutaneously. The staphylococci of mastitis appear to possess phages closely related to those of human staphylococci (Slanetz & Jawetz (72)), being single-type polyvalent phages producing small sharp-edged circular plaques. It is not possible by selective phage action to distinguish between human strains and animal strains. Palmer *et al.* (66) consider that staphylococcal bacteriophage is useful in doses of 10 ml. injected into the teat duct of dry heifers suffering from staphylococcal mastitis. It is of no value in milking cows, but in these cases staphylococcal toxoid injected intramusuclarly in doses of 10, 15 and 20 ml. at intervals of a few days is often of great value (Gould (73); Wilson (74)).

A further report on coliform mastitis in the Baltic States, where it appears to be of greater importance than in other areas, has been published by Kriisa (75) who records that 30% of 732 cases of mastitis were of this type. 118 cases were treated with intravenous injections of $CaCl_2$ and $MgCl_2$ and of antiserum, in addition to local treatment. Fifty-two cows recovered completely and fifty-two improved. Paresis occurred in some cases, and the prognosis in these was not good. Weirether *et al.* (55) treated four cases of coliform mastitis with Novoxil. One was completely cured but the other three were only temporarily improved.

Changes in the milk due to *Br. abortus* infection have been described by Hofstad (76). When large numbers of organisms are being eliminated from the udder the cell count, the chloride content and the brom-thymol blue test are affected. In naturally occurring cases there were no gross pathological changes, though histological examinations showed mononuclear infiltration of the interstitial tissue.

The changes in milk, especially the lower content of casein and solids-not-fat due to any type of mastitis, apparently affect cheese-making. Johns, Hicks & Gibson (77) state that such milk yields less cheese than normal, but that the quality of the product is not impaired.

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II. CONTAGIOUS ABORTION

Bacteriology

The relationship between the blood titre and the localization of *Br. abortus* in the organs of infected cattle has been investigated by Cordes (78) using cultural methods combined with animal inoculations. In five cows which reacted at 1:100 or less no *Brucella* could be found, but in seven where the titre was 1:200 or over *Brucella* were present in the udder. In six of these it was also found in the supramammary gland, in five in the milk, in four in the uterus, in three in the renal lymph nodes, in two in the lymphatics of the spleen and liver and in one in the iliac glands. It appears therefore that normally a titre of 1:100 or less indicates that an animal is not infective.

Although guinea-pigs do not die when infected with *Br. abortus*, Bergfeld (79) concludes from his experiments that the general health of the animals is seriously affected, more especially if the reticulo-endothelial system is blocked, when all tissue reactions are either more marked or occur more rapidly. The blood picture is that of a marked monocytosis with both a lympho- and leucopaenia, while epithelioid cells, especially in the Malpighian bodies of the spleen, are most marked during the second week after infection. This picture is similar to that described by Munger (see Huddleson (80)), who found that in human brucellosis there was a leucopaenia and relative lymphocytosis with slight monocytosis and an increase in epithelioid cells. In

guinea-pigs there was a reduction of 70% in the peripheral leucocyte count within 30 min. of the subcutaneous injection of a sublethal dose of the gluco-lipoid fraction of the bacillary bodies. This reduction was apparently due to actual destruction of the leucocytes by some lytic factor produced by the action of the substance on living tissues, since no such destruction occurred *in vitro*. This evidence does not agree with that previously reported in an earlier review by the present author (81).

Metzger & Stokes (82) passaged a virulent strain of *Brucella*, capable of producing macroscopic lesions in guinea-pigs, through a series of chick embryos. The original strain was kept concurrently on potato agar and remained smooth and CO_2 sensitive. After thirty-nine passages the strain was no longer CO_2 sensitive, after forty-eight it produced no lesions when injected into a guinea-pig intraperitoneally, while after the fiftieth transfer it had lost part of its ability to produce agglutinins on injection into susceptible animals. No R variants were found.

Ardrey (83), in a study of the factors affecting the isolation, cultivation and differentiation of *Brucella*, found that the addition of peptone to beef-liver infusion agar greatly improved this medium. Neo-peptone and proteose-peptone were found to be preferable to bacto-peptone. The largest number of colonies occurred on a medium prepared by Difco called Bacto tryptose agar (2% tryptose peptone, 0·5% NaCl, 0·1% dextrose and 2% agar). When dyes are added to the beef-liver infusion peptone medium a concentration of 1:700,000 is recommended for crystal violet and 1:100,000 for thionin and basic fuchsin. Occasionally the liver infusion in certain batches of media reduces the dye. The bacteriostatic action of both thionin and basic fuchsin is influenced by the *p*H and by the concentration of peptone in the medium. *Brucella* can also be cultured on simple basal media provided certain necessary factors are added. Koser, Breslove & Dorfmann (84) showed that a satisfactory medium consisted of amino acids, glucose and inorganic salts with thiamin, nicotinamide, pantothenic acid and biotin added. Of seven cultures, four grew slowly in the presence of nicotinamide and thiamin, and more rapidly if pantothenic acid was added, while the other three required biotin concentrate as well. Cocarboxylase and coenzyme 1 were of no greater value than thiamin and nicotinamide, while riboflavin, vitamin B_6, adenine, inositol and glutamine were without effect. A certain concentration of inorganic salts was necessary for growth, presumably owing to osmotic effects. The optimum concentration of NaCl was between 0·6 and 1·0%, while no growth occurred below 0·1%. KCl, Na_2SO_4 and K_2SO_4 could be substituted for the NaCl.

Ithurrat (85) found that he was able to induce strains of *Brucella* to produce S colonies on solid media and R cultures in liquid media. Smooth cultures of all three types of *Brucella* have been shown by Huddleson (80, 83) to possess a definite capsule. The technique he recommends is as follows: Smear a dilute suspension of *Brucella* cells on a slide and allow to dry in air. Add one drop of Keuffle and Essen drawing ink, no. 3000, and allow this to dry. Treat with methyl alcohol to which has been added 0·1% glacial acetic acid, and when dry stain with half-strength aniline crystal violet for 5 min. Wash with distilled water and dry. The capsule possessed by the S forms may be antigenic, since when Priestley (86) prepared extracts of S and R forms of *Br. abortus* using the trichloracetic acid technique, he found that the S extract reacted with antisera, was antigenic, heat stable, precipitated with acetone and was not dialysable. It was slightly toxic but did not give a skin reaction in guinea-pigs. The extract could only be prepared from S forms: the R extracts proved inert. The S strains of all types of *Br. abortus* also have similar electrophoretic mobilities. These mobilities remain constant during long periods of cultivation and are not affected within 4 months by storage in the cold in 0·2% formol saline. If the diluent is 0·5% phenol saline a decrease in mobility occurs, however, after 2 weeks (Stearns & Roepke (87)).

Serology

An attempt to determine the fundamental factors underlying the agglutination test has been made by Stahl & Pennell (88). They have shown that the condition for the maximum precipitation of *Brucella* antibody, i.e. for true equilibrium, varies according to the animal from which the reacting serum is obtained. Thus for human serum the optimum reaction occurs in mixtures held at 4° C. for 48 hr.; for guinea-pig serum 37° C. for 24 hr.; for goat serum 37° C. for 2 hr. or 4° C. for 22 hr.; for bovine serum 37° C. for 2 hr. or 4° C. for 22 hr.; for purified cow serum

and lipoid antigen 4° C. for 48 hr. The greatest amount of *Brucella* agglutinin substance was removed under the following conditions: (1) human serum, 37° C. for 48 hr., (2) guinea-pig serum, 4° C. for 24 hr., (3) cow or goat serum, 4° C. for 48 hr.

The agglutination test is not completely specific for *Br. abortus*. In addition to cross-agglutination with other members of the *Brucella* group there is cross-agglutination with *Proteus* and *tularense* antigen. This was studied by Calder (89) who, however, experienced no difficulty in detecting whether the patient was suffering from brucellosis or from either of the other diseases. In *Brucella* patients the titre to *Pasteurella tularensis* was always very low, and even though the Weil-Felix titre with O *Proteus* agglutinins was usually higher than the *Brucella* titre it did not rise during successive tests, whereas were the disease to be typhus a rapid increase in the titre would occur. In cattle the blood titre often drops at the time of abortion and may even be negative, although *Br. abortus* can be isolated from the placenta. Olson (90) compared the results of blood tests of 1110 cases of abortion with the findings recorded by microscopical examination of the placentae. The blood test was positive in 288 cases and examination of the placentae in 267. The number of cases in which the latter was positive and the former negative was only 1·4%, but Olson suggests that both tests should be used for routine laboratory diagnosis.

Considerable work has been done in America on the whole-blood agglutination test and the factors which affect it. Roepke & Fitch (91) consider that soluble material absorbed from the medium is of great importance in affecting the relative sensibility of the antigen; e.g. if 0·5% phenol saline is the suspensor, all the phenol is absorbed by the medium washed off with the bacterial cells. They suggest the use of large volumes of phenol saline to wash the organisms off the medium, followed by centrifuging and rewashing with phenol saline. Marsh (92) agrees with the necessity for washing the antigen but found it necessary to use a mixture of five strains of known antigenicity in order to obtain suspensions of constant sensibility. He added 5% of a 1·5% freshly prepared aqueous solution of crystal violet to his suspension and found that this had the same effect as 0·4% of gelatin. Roepke & Murdoch (93) continued the former author's earlier work and attempted to standardize the actual technique of the test itself. They tested forty-five sera of the intermediate type and their conclusions were as follows: (1) The position of the serum was of little importance, i.e. whether it is the first or the last of a series to be mixed with suspension. (2) Stirring for 4–6 sec. was necessary. (3) The spot size should vary with the dilution of serum from 34 mm. for 1:25 dilution to 17 mm. for the 1:400 (this keeps the depth of fluid constant). (4) The time of reading, i.e. the interval between the mixing and reading must be constant, especially if the plate is rotated. They suggest rotating the plate 5 min. after mixing the serum and suspension and reading at 10 min.

A number of workers have compared the results of several tests or of different modifications of the same test. Haupt (94) tested 229 sera by the agglutination test using two methods of setting up the test and two different suspensions. He recommends phenol-killed, and not heat-killed, suspensions and the adding of the suspensions directly to a small volume of undiluted serum. The tubes should be incubated at 37° C. overnight but not read till after a further 24 hr. at room temperature. While these results apply only to the particular suspension used, it may be noted that by his variations in technique he was able to obtain readings with the same serum varying by as much as four tubes. Weis (95) compared the ordinary tube agglutination test with the fresh-milk rapid test, the rapid-blood method and a modification of the latter. He found the milk test to give too many false negative results and the two rapid-blood methods too many false positives. He therefore considers the ordinary tube agglutination method to be the most reliable. Rislakki (96) agrees, having compared this test with Meinicke's flocculation test and the complement-fixation test, though he found these to be valuable in freshly infected herds. He suggests the use of Meinicke's test as a check for all doubtful reactors to the ordinary agglutination test. The same tests were compared by Hecke (97) who, however, reverses Rislakki's recommendations. He considers that Meinicke's reaction is the method of choice, using the agglutination test only in doubtful reactors. His main reason seems to be doubt as to what constitutes the critical titre in the ordinary tube test. If this is taken as 1:25, then of 1000 tests he found 4% disagreement between Meinicke's test and the tube test and 6% between the latter and the complement-fixation test, while if the diagnostic titre in the tube test was 1:100

the disagreement was 11 and 12% respectively. The flocculation (Meinicke's) test and the C.F. test agreed, on the other hand, to within 3%. Jersild (98) considers that the opsonin test is more sensitive than either the agglutination or the C.F. tests. He uses citrated inactivated blood instead of serum, and states that phagocytosis can be observed at a very early stage of the disease. If there is a small opsonic index this is markedly raised by the injection of brucellin for the skin test. The latter test has been favourably compared with the agglutination test by Dorofeev (99) who compared the results of 79,049 tests on 25,150 animals using both the intradermal and agglutination tests. There was fair agreement, but in young infected stock the intradermal test showed five times the reactors revealed by the agglutination test. The milk or whey agglutination tests are considered by Vellisto (100) to be reliable guides as to whether or not an udder is excreting *Brucella*, but he takes as his critical titre 1:2·5, which is so low that non-specific sedimentation is liable to occur. As he points out, this is particularly likely in summer samples or in samples from recently calved cows. In addition to the agglutinins in the milk it is possible that antibodies are also present in the urine of heavily infected animals, since Pennell (101) has shown that the urine of human patients contains small amounts of antibody which can be concentrated by dialysis and peptic digestion.

According to Todd & Ranney (102) a factor influencing the titre for *Brucella* antibodies is the absorption of agglutinins from the milk fed to calves. Pasteurization of the milk at 143° F. for 30 min. did not affect the agglutination titre of the whey but heating to 164° F. for 30 min. destroyed the agglutinins. Any reaction shown by calves due to ingestion of pasteurized milk containing antibodies would be purely temporary. It might be supposed, however, that if the milk fed is actually infected the calves might become actively immunized. Seelemann & Langeloh (103) confirm the usually held view that such immunization (by infection) is rare in young calves. They fed infected milk and found that only 5·5% of the calves reacted positively to Meinicke's flocculation test and these for only a few months. Of the calves born to reacting dams 50% reacted to the flocculation test but ceased to react by the seventh month.

Spicer (104) has suggested that the actual cause of abortion in animals infected with *Br. abortus* is lack of vitamin E. He bases this suggestion on the results of feeding rice bran to fifteen herds in which he markedly reduced the number of abortions. Unfortunately, no controls were kept. The difficulty of differentiating between abortions due to *Brucella* and those due to vitamin deficiency is emphasized by Hart & Guilbert (105), who found that normal foetal livers contained from 5 to 100 blue units of vitamin A per gram, whereas in thirteen foetuses aborted owing to *Br. abortus* infection the amount was below 5 blue units, in seven being nil. These results indicate either that *Br. abortus* impairs the transfer of vitamin A from dam to foetus, or that vitamin deficiency injures the placental cells and thus aids *Br. abortus* infection. The latter theory appears unlikely in view of the epidemiology of the disease. The authors point out that *Br. abortus* infection should be ruled out as the cause of an abortion before vitamin deficiency is diagnosed even though no vitamin can be demonstrated in the foetal liver. Against the theory of vitamin deficiency being the actual cause of abortion is the work of Cameron, Hughes & Gregory (106) who studied the genetic resistance of pigs to infection with *Br. abortus*. A non-reacting boar was mated to two sows which also could not be made to react to the agglutination test or become carriers by the oral administration of *Br. abortus* cultures. Thirty-three of their progeny were given 5 ml. of a heavy suspension of culture of *Br. abortus*, and a high proportion failed to develop any titre. When examined post-mortem, suceptible pigs showed *Br. abortus* in all lymph nodes, while of the resistant group of seventeen only three showed any *Br. abortus*, and this consisted merely of a few colonies isolated from the submaxillary glands. The authors conclude that there are resistant races of pigs and that these animals do not become carriers.

Control

During the past two years there has been a general tendency in both Britain and America to change from the policy of attempting to establish abortion-free herds to one of vaccination. In Britain this is due to the high incidence of the disease and to the great difficulty of keeping herds which have been cleared of infection permanently free. Such herds are particularly susceptible to epidemics of abortion due to the reintroduction of infection from outside the farm by fomite transmission or by other animals such as rats and dogs. The loss so sustained is

so great that few farmers are prepared to clean their herds, most preferring to have abortion endemic on the farm. In America the main difficulty appears to be that replacement cattle are often infected. The National Scheme introduced in Britain for the control of abortion (1) recommends blood testing and segregation in only a minority of herds. The official suggestion is that, in view of the necessity of repeated blood tests and the great risk of reintroducing the disease, only in those herds which are lightly infected and in which the system of herd management is especially favourable should eradication be attempted. All others should be vaccinated. In herds which are licensed to produce certified or tuberculin-tested milk a laboratory report certifying the presence of contagious abortion on the farm must be submitted before the vaccine may be used. This report is based on the tube agglutination test. In other herds it is sufficient for the veterinary surgeon to be satisfied from clinical evidence and previous history that contagious abortion is present in the herd.

There are two vaccines available under the scheme. Both are provided by the Ministry of Agriculture and no others are permitted. The vaccines are prepared from the American Strain 19 and MacEwen's strain 45/20 (see Vaccination below). The former is recommended as a single dose for calfhood vaccination and the latter as two doses for adult animals. The following general hygienic principles are laid down in the scheme:

(1) Report all abortions to the veterinary surgeon.

(2) Isolate calving animals and do not allow infected animals to return to the milking line till all discharges have ceased.

(3) Destroy any aborted calf and membranes and disinfect the premises.

(4) Feed aborted animals after the healthy stock and avoid contamination of food and bedding by attendants' boots.

This scheme has only been in operation since June 1942 and no results will therefore be available for some years.

In Germany the prohibition against live vaccination has also been withdrawn and the vaccine specified to be used is the bile culture vaccine of the Behring company (107). The herds injected must be infected, and the work is under the supervision of the local veterinary officials and research officers.

That some good should result from the British scheme is shown by the figures given by Knapp (108) for Florida. In this state the testing of cattle for contagious abortion is compulsory, and the percentage infection has fallen from 22·9 in 1934 to 0·99 in 1939. There has also been a marked increase in the production of milk. In one area the increase amounted to half a gallon per cow per day.

In America the Federal Bang Disease Control Scheme in 1940 contained 1,600,000 herds with 12,300,000 cattle. Of this number 55,000 herds with 1,100,000 cattle were officially disease free (109). It will be seen that the average number of beasts in each herd in the scheme is eight. In the cleared herds, however, the number of cattle per head is twenty. This seems to show either that large herds are easier to free than small or that more prosperous owners are better able to afford the cost of the precautions. In the whole of the U.S.A. there were 391 counties in twenty-three states officially described as 'modified accredited': of these ninety-three were added during 1941. A further 200 counties were being tested and thirty-three states had incorporated calfhood vaccination in the scheme (110). The methods used are: (1) Immediate slaughter of all reactors with indemnity. (2) Ditto plus calfhood vaccination. (3) Gradual elimination of reactors with calfhood vaccination but no indemnity.

The control of the disease was started in the twelve southern states in 1934 (Knapp (111)), the average infection rate at that time being 6·9%. By 1940 the incidence had been lowered to 3·1%. In eight of the states the test and slaughter method was used, while in the other four calfhood vaccination was also undertaken.

The third method mentioned above, viz. gradual elimination of reactors, plus calfhood vaccination, appears, however, to be the most favoured. Rosenberger (112) states that in California the test and segregation policy was a complete failure. Vaccination of heifers gave indefinite results, but no spread of the disease has occurred from mixing such heifers with the free stock. By adding such heifers and gradually slaughtering reactors progress towards eradication is now being made.

In the free herds 0·6% of the vaccinated heifers and 4·4% of the non-vaccinated stock have become reactors. In the infected herds only 4% of the vaccinated heifers added to the herds have reacted. Bonynge (113) also found it impossible to free the herds in his area from contagious abortion by the test and removal policy. When clean heifers were added to such herds they proved very susceptible, but calfhood vaccination with Strain 19 has greatly improved the position. McGregor (114) found that in a large beef herd in Maryland no progress was made by the usual test and segregation method, but that a new herd had to be built up using negative heifers. These were allowed to run with their dams for 6–7 months. They were then tested three times at 30-day intervals, and if negative were drafted to the negative herd. Fitch, Boyd, Kelly & Bishop (115) consider that a negative herd can most rapidly and safely be built up by removing each calf from its dam at an early age (1 week) and rearing it with other calves away from the infected herd. They studied a herd of fifty-six cows built up on these lines from an original infected stock for ninety-five lactation periods, during which 257 milk samples and 93 placentae and colostrum samples were examined. In no case was *Br. abortus* isolated.

Cotton (116) also objects to the test and slaughter method, but his objection is based not on the difficulty of freeing a herd but on the fact that in Alabama of 368 doubtful reactors 253 became negative during a 2-year period. He considers that these animals had probably been slightly infected but had recovered. He therefore holds that all herd replacements should be isolated until they have been tested twice at a 2-month interval. He emphasizes the importance of disinfection and draws attention to the danger of fistulous withers in horses, though he minimizes the danger from pigs infected with *Br. suis*.

The test and slaughter policy is, however, still advocated by Mohler, Wight & Lash (117). They claim that, while satisfactory results were obtained when individual herds were cleared, area eradication schemes were even better. They estimate the infection rate in America to be about 10%, varying from state to state between 3 and 22%. Even where cattle are on free range the infection rate may be high (120). They state that in herds where eradication has been successful the animals are thriftier, there is a 25% increase in the number of calves, the milk yield is increased and there is a decrease in sterility and udder disease. They claim that 4–5 tests should result in the eradication of the disease. This view receives strong support from the work of Plastridge, Rettger & White (118), who found that of 149 infected farms 64·8% were freed in 1–2 tests, 22·7% in 3–4 tests and 12·7% after more than four tests. Their report covers 14 years' work, during which the annual number of tests rose from 2378 to 42,700 and the number of herds from 27 to 624. The number of negative herds rose from 4 to 473. The average incidence of infection at each herd when first tested was 25%, and it was found that more doubtful reactors became positive in the herds where the spread of infection was most rapid. Reinfection in herds in which eradication had been successful was usually due to infected cattle introduced into the herd, but some cases were due to animals breaking into or out of the pastures or to infected horses or pigs. In a few cases it was thought that persons going directly from an infected to a clean herd introduced infection into the latter.

The danger of the spread of abortion through foot-and-mouth disease convalescent serum has been pointed out by Kaiser (119), who found that *Brucella* could remain alive for 13 days in unpreserved serum and for 5 days in carbolized serum. He therefore advocates carbolizing the serum and then storing for 7 days. As to fomite transmission he found that all the usual disinfectants were quite satisfactory for destroying this organism, among those tested being lime, bleaching powder, cresol, phenol, corrosive sublimate, formalin, hypochlorite, chloramine and caustic soda.

According to Marsh & Welch (120) eradication of the disease from beef herds is relatively easy. They state that all such cattle in their area are bred to calve during a 60-day period ending on 1 June. Tests are taken 3–4 months after the birth of the last calf, and two annual tests should be sufficient. They advise that all replacement calves should be vaccinated. They draw attention to the fact that while the presence of a few reactors is normally of little significance in beef herds an outbreak of brucellosis is liable to occur if hay has to be fed presumably owing to the closer contact of the animals at the feeding places.

The need for some degree of herd immunity as the primary basis on which to build a free herd is stressed by Fritzsche (121), who considers that no attempt at elimination of reactors

should be made except during the so-called latent period of the disease, i.e. 1–2 years after an outbreak has occurred.

The spread of contagious abortion from farm to farm by animals other than cattle is still being studied by several workers. Bosworth (122) has continued his study of the rat as a factor in the transmission of the disease. He found only one rat out of 167 caught on *Brucella*-infected farms was carrying infection. Two cows fed on food contaminated by infected rats could not be shown to have contracted *Brucella* infection. Witte (123) incriminates the hare as a possible source of infection, having found the organisms in a wild hare. Ruhland & Huddleson (124) fed cockroaches and flies on cultures of *Br. abortus* and then squeezed anal contents on to tryptose-agar crystal-violet plates. The cockroaches were uniformly negative, but the flies were invariably positive up to the latest time examined (96 hr.). Tettenborn (125) tested 1790 sheep on eighteen farms where *Brucella* infection was endemic in the cattle, and found only one farm where the sheep reacted to the agglutination test and Meinicke's test. These particular sheep had been previously infected with *S. abortus-ovis* and by foot-and-mouth disease and were in a much weakened condition. Even in these no abortion due to *Brucella* occurred. There have been several investigations into the presence and control of brucellosis in sheep, especially in the Soviet Union, where it appears that *Br. melitensis* is the prevalent organism. Orlov & Karneeva (126) report favourably on the use of the skin test using Brucellysate (Zdrodovskii (127)). By segregating the young negative animals brucellosis was eradicated from thirteen farms in 2 years. It is emphasized that segregation of the older animals is of value as they consider that approximately 9% of 'carrier' sheep do not react to serological or allergic tests. Eremin (128) draws attention to the danger from sheep dogs which have eaten infected placentae, aborted lambs or the carcasses of newborn lambs used for astrakhan production. 28% of such dogs were found to react to the agglutination test and from two *Br. melitensis* was isolated. Feliks (129) demonstrated *Brucella* in the milk of recently lambed ewes, and he records the danger of infection to sheep from the urine of persons suffering from brucellosis (see Pennell (101)).

As regards brucellosis in horses, the general consensus of opinion is that the disease is more common in horses in rural than in urban districts and is especially noted in horses on *Brucella*-infected farms. Positive titres to the agglutination test are present in a high proportion of cases of 'poll evil' or 'fistulous withers', but no causal relationship seems to have been established between *Brucella* infection and periodic ophthalmia. *Br. abortus* was found in the faeces of two horses by Karlson & Boyd (130), and they stress the possibility of this method of spread from horses to cattle. Stone (131) records that he found the prozone phenomenon to be particularly marked with equine sera, and that in some cases it extends to the 1:200 dilution.

Vaccination

As noted above the official policy in Great Britain, Germany and America now favours vaccination rather than eradication, though the latter is still preferred in certain areas of the U.S.A. The vaccines used are mainly attenuated, the best known and most extensively employed being the American Strain 19. The care necessary in preparing and issuing such a vaccine for field use is emphasized by Mingle & Mathie (132), who found that anomalous results in the field were due to the issue of a vaccine containing an unusual dissociation form (termed W) which did not correspond to either the S or R forms or to any of the usual intermediate types. Similar variants were obtained from the old vaccines. They were not able to ferment carbohydrates but were able to tolerate the usual dyes used for differentiating this species. There were also serological differences between the parent and variant strains, but the W form readily reverted to the typical S form indistinguishable from Strain 19 when passaged through guinea-pigs.

The best vehicle in which to suspend the vaccine for injection was studied by Eichorn, Mingle & Murdoch (133), who compared the results obtained when Strain 19 was suspended in liquid paraffin, oil of sweet almond, lanolin, saponin, cholesterol and saline. The suspensor had no effect on the general reaction, the agglutination response or on the vaccine strain itself, but had a considerable effect on the local reaction produced. This reaction persisted for only 4 weeks when saline was used but for 5 months when 50% lanoline liquid paraffin was the suspensor. It is interesting to note that the authors found no correlation between the *in vitro* and *in vivo* tests. Thus the 50% lanoline liquid paraffin suspension was sterile after 96 hr. at 37° C. *in vitro*,

but *in vivo* unchanged Strain 19 could still be recovered from the vaccination site 85 days after injection.

It appears from the work of Sunderville (134) that Strain 19 does not generalize in the calf. In six negative calves vaccinated subcutaneously with 5 ml. of vaccine, blood cultures were negative by the 28th day, though the organism became localized in the non-pregnant genital tract of the calf. Madsen (135) has shown that calfhood vaccination with a virulent strain of *Br. abortus* protects most animals against a heavy infection before calving. Of seven calves vaccinated and then infected during gestation by conjunctival instillation of virulent culture only one aborted, though two were slightly early in calving. Five vaccinated controls all aborted. Although the vaccinated animals carried their calves to term they all showed *Br. abortus* in the placentae. A field trial with eighty-seven cows vaccinated and then exposed to natural infection resulted in eighty-two calving normally without a positive blood titre, four calving normally but with a high titre, and one aborting with an infected placenta. Delez (136) also tested the resistance of vaccinated animals to conjunctival instillation of *Brucella* suspension. He vaccinated nine calves at 4–6 months old and exposed them to infection during their second pregnancy by instilling a saline suspension of virulent *Br. abortus* into the conjunctival sac. Six calved normally, the other three calving live calves prematurely. *Br. abortus* was recovered from these animals. Of the four controls one aborted, one calved a premature live calf and two calved dead calves. The three examined all showed *Br. abortus*.

The field results with Strain 19 are equally encouraging, though Winter (137) reports relatively severe clinical reactions including two deaths in 1172 calves vaccinated. Nevertheless, he found that 80% of the calves became negative reactors within 12 months and that of 978 pregnancies only nine terminated in abortion due to *Br. abortus*. In 5 years five out of twenty-five herds were cleared of reactors in the adult stock and ten had only 1–3 reactors or suspects. Thomsen (138) states that of 328 vaccinated calves only 3·3% aborted, while of 158 unvaccinated 25% aborted. Tomkins (139) gives a statistical analysis of his results and concludes that calfhood vaccination may at the least be said to reduce susceptibility. Birch, Gilman & Stone (140) and Birch (141), comparing twenty-eight vaccinated animals and fourteen unvaccinated, found a significant difference in favour of the vaccinated animals for both the abortion rate (1·4% compared to 24·5% in the unvaccinated) and the reactor rate during the first and second pregnancies. The reactor rate was rising towards the end of the second pregnancy by which time 20% of the vaccinated animals were reactors. The conclusion drawn was that, while calfhood vaccination was useful for protection during the first and possibly second pregnancies, it was not of great value for later pregnancies. Mohler (142) and Mohler, Wight & O'Rear (143) vaccinated 17,000 calves in 260 herds scattered through twenty-four American states. 96% of the pregnancies were normal. Of these 83% were negative to the agglutination test, 5% were positive and 12% were suspicious. Of the animals which aborted (310) only 32% (128) were positive reactors. This number (128) is 1·6% of the whole vaccinated group. These figures therefore tend to show that calfhood vaccination does protect the animal through more than two lactations, since of the 30% original reactors in the herds 24% were still in the herds after 4 years, thus exposing the vaccinated animals to considerable natural infection. It is of course possible that by this means natural 'revaccination' of the animals occurred before the protection of the calfhood vaccination had diminished. Presumably the animals in eleven herds reported by Rabstein & Welsh (144) were also exposed to infection, since they state that all reactors were retained in the herds and that the initial average infection was 36%. Despite this they report that the blood titres rapidly became negative in the animals vaccinated when young. They divide their vaccinated animals into three groups. 642 vaccinated at 3–8 months old, 89 at 9–12 months and 65 at 13–21 months. There were 667 pregnancies and only five abortions due to *Br. abortus*. They could find no evidence that Strain 19 was transmitted to non-vaccinated animals, that it became established on the premises, that it increased in virulence by passage, or that it became established in the udder of vaccinated cattle. The length of time that a positive titre remains in the blood serum depends, according to Haring & Traum (145), not only on the age at vaccination and the effect of exposure to virulent infection, but also on the relative proportions of the S and R variants in the vaccine. They found that calfhood vaccination with this vaccine was of great value, since in a herd with 44% infection the disease was eliminated in 6 years.

Calfhood vaccination with Strain 19 has been adversely criticized by McEwan (146), who has collated the figures available for field experiments with this vaccine. Of 8182 vaccinated animals which calved 7872 produced normal calves and 1·6% aborted with a positive agglutination test. The agglutinin response had not returned to normal in 1346 of the animals, i.e. 17% were still positive after calving. This is a far higher proportion than would have been anticipated, and it would be interesting if the figures could have been further analysed to show the respective average ages at which the majority of the positive and negative animals were vaccinated. When he abstracted the figures for the second gestation he showed that vaccination had had no noticeable effect. In 1938, 9·6% of the vaccinated animals aborted in their second gestation against 12·9% of non-vaccinated animals. In 1939 the figures were 4·3 and 2·3% respectively.

In Britain, McEwan (147) has continued his work on avirulent strains as vaccines. He vaccinated one group of eighty-two guinea-pigs with a fully virulent CO_2-sensitive strain of *Br. abortus* and another group of seventy-three with an anaerobic rough strain of low virulence. At varying periods up to 12 weeks batches of guinea-pigs, together with adequate controls, were inoculated with falling doses of *Br. abortus* using a fully virulent aerobic strain. When killed 4 weeks later cultures were made from the spleens. The presence of the aerobic strain was held to indicate reinfection, and it was found that while the guinea-pigs vaccinated with the R strain were more resistant than the controls, the fully virulent vaccine had conferred a much greater resistance on the animals. A further series of experiments showed this resistance to be high within 1 week of injection and to be even higher after 24 weeks. McEwan argues that, if the reaction in cattle is analogous to that in guinea-pigs, vaccination with a culture of full virulence sets up a high resistance to reinfection, and that therefore any future abortion is due to persistence of the vaccine strain and not to fresh infection. Assuming this to be true, he suggests that vaccines of graded virulence be employed according to the degree of susceptibility of the animal to be vaccinated, as judged by age, condition of the mammary gland and the time interval before pregnancy. Working on the hypothesis that the degree of protection depends on the virulence of the vaccine, McEwan & Priestley (148) performed a series of experiments in guinea-pigs, using vaccines of graded virulence. They also wished to ascertain if a non-virulent vaccine when followed by a virulent vaccine prevented the persistence of the latter in the tissues. The results were inconclusive but they showed that (*a*) dead vaccines conferred some protection, (*b*) any form of live vaccine was preferable to dead vaccine, and (*c*) that vaccination with a strain of low virulence (45) followed by a second injection of a strain of greater virulence (45/20) gave the best protection. McEwan (149) carried this work further and found that if guinea-pigs were definitely infected by a virulent strain certain changes occurred in their response to injections to *Br. abortus*. They became very resistant to a normal infective dose and the bacteria were rapidly removed from the blood stream, though they did not localize in any of the body organs. If a sufficiently large dose of the organisms was injected intraperitoneally the animal died within 24 hr., being apparently sensitized to the bacterial protein, but if the dose, though large, was sublethal, characteristic changes occurred in the omentum. This organ changed from a membrane to a thickened band of tissue often studded with abscesses. Such changes may be regarded as a local immunity response since, if an infected guinea-pig was inoculated in the thigh, a local abscess was formed in the popliteal area—as distinct from the lack of local reaction in a non-infected guinea-pig. These changes are only related to previous infection, since dead vaccines or injections of organisms of reduced virulence do not produce them.

McEwan's work has been directed towards finding a laboratory method for testing the virulence of a vaccine for cattle and also determining the maximum virulence permissible in vaccine strains, i.e. a virulence which, while producing immunity, is not sufficient to cause the organism to localize in the uterus or mammary gland. He had already demonstrated (150) that the virulence of a strain was similar in guinea-pigs and cattle and could be more or less rapidly enhanced by passage. Thus his strain 45, which was practically avirulent and unable to infect cattle, could, after twenty passages through guinea-pigs, infect and cause abortion in pregnant animals. It could also cross-infect susceptible cows by contact with vaccinated animals. It appears, however, that it is not capable of infecting non-pregnant animals. For instance, thirty-three such animals in an abortion-free herd were injected with three successive doses of this vaccine (45/20).

None produced agglutinins or became permanently infected (151). McEwan (146) has summarized the position of vaccination and has laid down the following desiderata for any vaccine:

(1) It must confer a satisfactory immunity.
(2) It must remain constant in its properties.
(3) It must not infect the type of animal to be vaccinated.
(4) It must not produce agglutinin response.

As noted above, he criticizes Strain 19 on the grounds that it is doubtful if it confers a satisfactory immunity, at any rate when used as a single calfhood injection; it is not known to remain constant in its properties, and it infects the vaccinated animals and produces agglutinins. He therefore suggests the use of his vaccine 45/20 as it fulfils all the criteria above and advises reinjection after each calving. This vaccine is, in fact, being issued by the Ministry of Agriculture for use in adult stock.

In sharp contradistinction to McEwan's strictly controlled vaccine, are the many vaccines at present sold commercially in this country. While some are of value and may be standardized by the sellers, others are apparently of little value. Rowlands & Harbour (152) compared and examined eight live vaccines from different sources. The virulence varied considerably and two of the vaccines were contaminated, one containing no living *Br. abortus* but only contaminants!

Chemotherapeutic treatment is not meantime being extensively studied, the early interest in the use of sulphonamides having died down as their ineffectiveness became obvious. Two negative reports on other methods have been published. One by Zeller & Henninger (153) unfavourably criticizes treatment with 'Serobortan', a product of horse serum, combined with vaccine and also with 'AO_3', a mixture of camphor and the sodium salt of *o*-indophenol. In no case did either method cure an infected cow. The other, by McEwan & Oxspring (154), states that 'Abortulin' failed to cure two cows in which *Br. abortus* was continually isolated from the milk.

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III. TUBERCULOSIS

Bacteriology

The reaction of the tubercle bacillus to different stains and staining methods has been studied by Larsen (155), using Hagemann's fluorescent method, Rochese's homogenization method, Halberg's 'light blue' stain and the ordinary Ziehl-Neelson stain. The last named gave the poorest and the fluorescent method the best results, except with stomach washings for which it was useless. These results are explained by Richards's (156) work. This author melted mycolic acid (the only acid-fast component of *Myco. tuberculosis* so far isolated) on slides and stained it with auramin and carbol-fuchsin. The auramin stained the mycolic acid yellow, and this colour resisted decolorization in acid alcohol for 72 hr. The colour produced by carbol-fuchsin was, however, easily decolorized. Mycolic acid itself is only weakly fluorescent in ultra-violet light, but when stained with carbol auramin an intense yellow fluorescence is produced. A method of identification of the tubercle bacillus, which involves staining combined with cultural methods, has been advocated by Pryce (157). Suspected material is smeared on Petri dishes or glass slides within a ring of bakelite, the smears are dried, treated with sulphuric acid to remove contaminants, and a haemolysed citrated blood medium poured over them. After a few days' incubation micro-colonies are formed, and these can be stained by Ziehl-Neelsen stain and observed under the microscope. Another cultural method has been suggested by Steenken & Smith (158), who added malachite green in concentrations ranging from 1:1000 to 1:10,000 to the digested sediments from 114 specimens of sputum, fifty-one of urine, twenty-eight of pleural fluid and fifty-seven of gastric contents. The degree of contamination of cultures grown on the authors' modification of Hohn's medium (a lacmoid medium) was greatly reduced by this process and growth appeared earlier. Still another technique for the isolation of *Myco. tuberculosis* has been described by Boquet & Lenci (159). They added 9 ml. of a 4% saline solution to 1 ml. of the tuberculous material. This was thoroughly mixed with a pipette and put in the 37° C. incubator for 30 min., being shaken at intervals. It was then diluted with distilled water in decimal dilutions from 1:10 to 1:1000 and 0·1 ml. of each dilution spread evenly over the

surface of Loewenstein's or Petragnani's media. After 6–8 weeks' incubation the colonies were counted. The method is claimed to be particularly valuable for following the course of the disease. Sahai (160) examined the growth of forty-five strains of human origin, sixty-nine of bovine and fifty of avian on Lowenstein's, Petragnani's, Petroff's, Sweany and Evanoff's, and Herrold's and Holth's media. Loewenstein's medium was consistently the most successful.

Sasano, Caldwell, Needham & Medlar (161) also consider that cultural methods are reliable for the diagnosis of tubercle and have summarized their findings in cultural examinations from 100 consecutive tuberculous guinea-pigs. They suggest that a combination of Petragnani's and Bordet-Gengou's media gives the most reliable results when only a few tubercle bacilli are present.

Pasricha, Panza & Banarjee (162) consider, however, that intracutaneous inoculation in guinea-pigs is the most reliable method of diagnosing tuberculosis. They point out that not only is the result obtained more rapidly (in 7–14 days) than by other methods, but that at least four separate samples can be tested in any one guinea-pig, the only condition being that the sites chosen for inoculation must be drained by different lymph channels. Diagnosis is by scrapings from the ulcer.

Another factor of importance in culturing the tubercle bacillus is the CO_2 pressure. This appears to have a specific effect on the growth. Davies (163) has shown that optimum growth with the human strain H37 was obtained in the presence of 2·5% CO_2 in equilibrium with 0·0125 *M* $NaHCO_3$ at *p*H 7·4.

The methods of differentiating the various types of tubercle bacillus *in vitro* have not changed during the period under review, but there is some evidence that the bovine type may be more common in human patients than had previously been supposed. Munro (164) examined 1160 cases of pulmonary tuberculosis and found that fifty-eight (5%) were due to the bovine type. He points out that the virulence of this type appears to be equal to that of the human type, since twenty-two out of thirty-eight females and twelve out of twenty males died from the disease. Griffith (165) confirmed these results, finding thirty-one out of 515 cases of pulmonary tuberculosis (6%) to be due to typical bovine strains. The bovine type has also been incriminated in a much higher proportion of cases of non-pulmonary tuberculosis. Mushatt (166) found that in some fifty cases which occurred in children under 14 in Eire, ten out of fourteen cervical gland cases, four out of twenty-two bone and joint cases, and three out of twelve meningitis cases were due to the bovine type. That this type is present in a larger proportion of cases than is usually recognized is also suggested by Gróh & Remete (167) who examined forty-nine cases of renal and four of testicular tuberculosis: using the ordinary methods of differentiation forty-three of the specimens showed the bovine type of bacillus, but when Gróh's own method was used all showed this type. The explanation is stated to be that in the ordinary methods of culture the more rapidly growing human type overgrows the bovine in any mixed culture. Gróh's method is to use Loewenstein's medium for primary cultures. The young colonies are examined under a lens ($\times 10$) and smears made. These smears are examined and the human and bovine types differentiated on morphological grounds. Colonies with a high proportion of bovine bacilli are subcultured and inoculated into rabbits and guinea-pigs. By this method the author claims to have demonstrated that most cases of non-pulmonary tuberculosis in man are due to the bovine type. Holth (168) has recorded what he regards as genuine instances of transformation of the human type to the bovine and vice versa. It is possible that, if Gróh's suggestion is correct, the real explanation is that Holth's original cultures were mixed and that he had picked colonies containing a preponderance of one type. Holth recommends the use of a special medium consisting of 90 parts of whole egg, 60 of potato filtrate, 3 of tuberculin and 5 of 5% lactic or hydrochloric acid. Colonies appear on this medium in 3–4 days. It does not differentiate between the types. Griffiths (169) also refers to a change of type in cultures of six strains of mammalian tubercle bacilli. Comprehensive tests indicated that they had changed to the avian type. He did not, however, consider that true modification had occurred but that the apparent changes were accidental.

Pathology

The series of articles by Innes (170) on the pathogenesis of tuberculosis has now been completed. His last article does not lend itself to abstraction, and interested readers should consult the

original. Hermansson (171) has studied congenital infection in the calf by examining post-mortem forty-two cases of uterine tuberculosis in the cow. All cows were affected in organs other than the uterus: in thirteen there was generalized, in twenty-five pulmonary, in thirty pleural, in twenty-seven peritoneal and in eight udder tuberculosis. In twenty-three cases there were tuberculous necrotic lesions of the wall of the uterus and in nineteen the cotyledons were grossly affected. Only nine of the foetuses were not affected with the disease, and in all these the dams were less than 5 months pregnant. All the remaining thirty-three were affected in the hepatic lymph nodes; twenty were also infected in the mediastinal nodes, fourteen in the bronchial nodes, twelve in the liver and two in the lungs. Hermansson considers that pregnancy must occur before or just after uterine tuberculosis becomes established and that the calf becomes infected via the placenta, the resulting spread of infection being either haematogenous or direct from the peritoneum. White & Minett (172) studied the pathogenesis in young calves artificially infected. In their first experiment four calves from 5 to 7 days old (all from a tubercle-free herd) were fed fourteen daily doses of raw milk containing sufficient tubercle bacilli to infect guinea-pigs. Two of the calves were bucket fed, the other two were fed by stomach tube. One of the calves fed by bucket and one fed by tube developed caseous pneumonic tuberculosis, this being attributed to primary aspiration infection of the mediastinal lymph nodes. The other two did not develop the disease. Four further calves were similarly fed with three half-pint doses of heavily contaminated milk. Three developed the disease. One fed by stomach tube showed tuberculosis of the tonsil with a single lesion in the bowel, while the other developed pulmonary tuberculosis. Of the two fed by bucket only one became infected, showing caseous bronchopneumonia with caseation of the pulmonary and mesenteric lymph nodes. A teat bucket was then used to feed three more calves with infected milk; two developed alimentary tuberculosis and one respiratory. Intratracheal injections of cultures failed to set up tuberculous pneumonia in three calves, although local lesions at the site of the injection and of the associated lymph nodes was recorded. The authors then incised the abdominal wall and the abomasum in ten calves and introduced a culture of tubercle bacilli enclosed in a capsule. This was passed into the duodenum. Three of the calves died and a further two failed to show tuberculous lesions. Three of the remaining five developed tuberculosis of the abomasal wound, one also showing miliary tuberculosis of the lungs. The other two calves showed tuberculous Peyers patches and associated lymph nodes.

According to Fichtner (173) the usual seat of the alimentary lesion in natural cases is the ileum and the distal end of the jejunum. This author found that in twenty-one of fifty-six cases of chronic pulmonary tuberculosis in cattle there was also a tuberculous ulceration of the intestine. He states that intestinal ulceration is rare in early generalization, that the associated lymph nodes are invariably affected wherever there is intestinal ulceration, and that he failed to find tubercle bacilli by microscopical examination in any intestine which appeared normal. Aerogenic infection appears, however, to be the most common form of tuberculosis. This was the cause of 520 cases out of 1840 cattle slaughtered at Cologne and examined by Thewes (174). An incomplete primary complex was usually present in the pulmonary lymph nodes. The liver was the most frequent abdominal organ involved. Weers (175) also states that aerogenic infection is the most common form of tuberculosis in cattle and states that in 44% of his cases the primary focus was pulmonary.

The pathogenesis of the actual lesion has been studied by several authors. Pagel (176) made histological examinations of the lesions produced in mice, guinea-pigs and rabbits by the intracutaneous injection of 1 mg. of bovine, human and avian types suspended in saline. The typical reaction in mice (which are resistant to tuberculosis) was a marked proliferation of large mononuclear cells, filled with lipoid (foam cells) containing tubercle bacilli which had lost their basophilic characters and were eosinophile. True mononuclear cells, and tubercle formation were not observed, and the lesions were small. In the more susceptible rabbits and guinea-pigs, however, true tubercles were formed. Mononuclear cells were abundant and foam cells absent, while the bacilli were basophile and not eosinophile.

The pathogenicity of various strains may possibly be evaluated by inoculation of chick embryos, since Emmart & Smith (177) have shown that the extent of tubercle development in the chorio-allontoic membrane is dependent on the virulence of the strain injected, no tubercles

being produced by heat-killed bacteria or by tuberculin. These authors are studying the value of this method as a means of assessing the tuberculocidal action of drugs.

The action of the various types of tubercle bacilli on different classes of animals has been further studied. Griffith (178) infected hamsters by subcutaneous inoculation and by feeding. He states that the human strains produced generalized lesions when injected, but either failed to infect or produced minimal lesions when fed. Avian bacilli were less virulent, though multiplication of the bacteria in the organs was noted. The bovine type was the most virulent, producing generalized lesions even when fed. The vole bacilli were found to give rise to widespread epithelioid lesions developing very slowly and leading to caseation. Griffith (179) also injected the different types of tubercle bacilli into toads and found that they disseminated throughout this animal but failed to produce visible lesions. The strains could be recovered for periods up to 3 years without any modification in their original characters. In another article (180) this author has shown that the grass-snake is highly susceptible not only to reptilian strains but also to avian.

The rat appears to be slightly more susceptible to bovine than to human strains. Oswald (181) injected these animals intravenously with both types and then examined the lungs, livers and spleens. He found that direct microscopic examination of sections was unsatisfactory, since it did not determine the numbers of living bacilli and completely failed to demonstrate their presence when only few were present. He therefore counted the colonies in cultures from weighed amounts of each tissue. By this method he demonstrated that bovine bacilli multiply more rapidly and reach greater numbers in the lungs than do human bacilli, though the latter did increase slowly. The multiplication of those bacilli which localize in the liver and spleen was markedly less and appeared to be inhibited so that after 6 months the number of colonies obtained was generally less than at earlier periods.

Wessel (182) also examined the reaction of the rat to the different strains of tubercle bacilli. He agrees that the bovine type is the most pathogenic for rats, but states that both the lungs and the kidneys destroy even this type of bacillus. He found that tuberculous lesions without caseation occurred in all the visceral organs when either mammalian or avian strains were injected intravenously. He notes that, even though lesions were present, the animals showed no symptoms of disease and continued to gain in weight. White mice have also been used to differentiate the various types of bacilli (Stamatin & Stamatin (183)). The mammalian could be distinguished from the avian by the length of time elapsing before death after an intravenous injection of 0·01–0·1 mg. of bacilli. Mammalian strains caused death within 50 days, whereas with the avian type death did not occur before 80 days. Moreover, the avian type localized in the spleen and liver, while the human produced massive lesions in the lungs. Intraperitoneal injections were not satisfactory for differentiation.

Massive pulmonary caseation in rabbits was found by Saenz (184) to be produced not only by living virulent bovine-type bacilli but by dead organisms of this type if injected in liquid paraffin. Human-type bacilli did not cause this reaction and produced only a few non-lethal foci in the lungs. Avian bacilli were intermediate. Saenz & Urquijo (185) continued this work using B.C.G. They found that the lesions produced by dead bacilli of this strain suspended in liquid paraffin were more severe than those produced by the living organisms if injected without paraffin, but were less extensive than the lesions following the injection of dead bovine bacilli in paraffin. The same technique was used by Westwater (186) in his attempts to demonstrate whether antibodies are produced at the site of inoculation or are central in origin. He inoculated a series of rabbits in the skin with heat-killed bacilli suspended in liquid paraffin and excised the areas at varying periods from 1 min. up to 14 days after inoculation. Complement-fixation tests were made on the sera of these rabbits and on the controls from which the area had not been excised. The greatest titres were in those in which the area had either not been excised or where excision had been delayed till at least 30 hr. after inoculation. If the site was excised within an hour no antibodies could be demonstrated, but if excision was delayed for 6 hr. they were demonstrable. Further experiments showed that antibodies (complement fixation) appeared earlier in the lesion at the site of inoculation than in the blood serum, and that in extracts of the local lesion the antibody concentration was higher than in the blood stream for about the first 3 weeks, though subsequently it was greater in the blood serum. Another

development of the paraffin technique has been reported by Freund, Casals-Ariet & Genghof (187), who repeatedly injected rabbits with constant doses of heat-killed tubercle bacilli but varied the amount of liquid paraffin in which the dose was suspended from 0·0001 to 0·3 ml. They found that the degree of sensitivity and of antibody formation was directly proportional to the size of the lesion produced, i.e. to the size of the dose of liquid paraffin.

An attempt to ascertain the actual mechanism by which liquid paraffin increases the pathogenic powers of dead tubercle bacilli was made by Choucroun (188) who suspended bacilli in paraffin and then centrifuged till all bacterial debris was removed. The supernatant liquid contained an active principle which, when injected, sensitized animals to tuberculin. From this supernatant liquid a chemical substance was obtained, free from any bacillary debris, which produced lesions identical with those produced by the injection of dead tubercle bacilli in paraffin. When 2 mg. of this substance was suspended in paraffin and inoculated intraperitoneally into guinea-pigs, typical lesions of pulmonary tuberculosis were caused though no bacillary bodies were present either in the inoculum or in the lesions.

In guinea-pigs and also in humans the actual blood picture is said by Binda & Urquia (189) to be altered by tuberculosis. They state that the polymorphonuclear leucocytes change to a peculiar type of granular leucocyte from 9 to 12μ in diameter and packed with large granules of varying size arranged round the periphery of the cell. The usual percentage of this type of cell is 30–50, but in extreme cases it may rise to 98–100. Turley & Dougherty (190) state that there is a relative neutrophile leucocytosis at the beginning of a tuberculous infection and also just before death, but that the real response of an animal to infection with this organism is a lymphocytosis, the magnitude of which varies according to the degree of resistance to the disease. They assert that these cells play a specific part in the general resistance of the animal body to the proliferation of the organisms, and that this role is due to the activity of the living cells and not to any extracellular product. The argument they use is that the cells dispose of, or neutralize, the bacterial toxin and thus decrease the toxic properties. They point out that the greatest proliferation of tubercle bacilli occurs in the lymphatic glands which is, in their opinion, due to the action of the lymphocytes in neutralizing the waste metabolites of the bacteria and thus removing an inhibitory factor on the growth of the organism. The importance of the mononuclear lymphocytes in the resistance of an animal to tuberculosis has been confirmed by Lurie (191), who has studied the genetic resistance of families of rabbits to this disease. He bred eight distinct strains of rabbits by constant brother-sister matings. Each generation was tested for resistance to natural respiratory tuberculosis by exposure in special cages adjoining a common run in which a number of rabbits injected intravenously with a small dose of bovine tubercle bacilli were confined. The caged rabbits were thus exposed to equal chances of infection by the urine, faeces and nasal discharges of the infected rabbits. The course of the disease in the caged rabbits was followed by radiological examinations of the chest, by intradermal tuberculin tests, and by careful post-mortem records, particular attention being paid to the site and progress of the primary focus. They divided their animals into three groups, resistant, intermediate, and susceptible, and found that certain families could be assigned to each group. Thus in animals belonging to the resistant families the disease was a localized chronic ulcerative phthisis of the reinfective type, while in susceptible families there was a rapidly progressive generalized disease. Histological examination of the lungs suggested that the resistance shown by certain strains was largely due to the ability of the mononuclears of these animals to inhibit the growth of the bacilli. A similar result was obtained when animals from the different families were tested by the inoculation of uniform quantities of the same suspension of bacilli, the degree of resistance being measured by the mean survival rate. This was significantly longer in the animals of the resistant families. Further work in collaboration with Zapposodi (192) showed that in animals of the resistant families the inoculation of killed tubercle bacilli caused a rapid development of the allergic state and local immunity with a high agglutinin titre. The response in susceptible families was much slower. It was noted in respect of local immunity that the spread of indian ink injected into the skin bore an inverse relationship to the period of survival of the animal.

The effect of the diet on the resistance to tuberculosis has been investigated by Vetukhov (193) who suggests that increasing the minerals in the diet increases the resistance to disease. Unfortunately, the original is in Russian and his evidence cannot be checked.

Mills, Barth & Gunn (194) determined the sedimentation rates of the red blood cells following bronchial inoculation and found that a rapid increase in the sedimentation rate meant that the tubercular lesion in the lung was rapidly extending and thus the sedimentation rate was a guide to the morbid process.

It appears from Kluge's work (195) that not only is the spread of the bacillus usually lymphatic but that it is actually absent from the blood stream. In none of thirty-eight artificially infected guinea-pigs could tubercle bacilli be isolated from the circulating blood nor could the bacilli be isolated from the carcass blood of six examined post-mortem. Similar results were recorded for nine fowls and for the circulating blood of two tuberculous cows tested daily for 42 and 7 days respectively previous to natural death. The carcass blood of these two cows was, however, positive, as was the musculature when examined after death.

Yegian (196) has found that even when tubercle bacilli are injected directly into the hearts of the guinea-pigs the bacilli, though setting up generalized tuberculosis, are rapidly eliminated through the kidneys, samples of urine being positive (by animal inoculation) within 10–50 min. of intracardiac injection.

Control

The report of the Tuberculosis Committee (197) states that in the U.S.A. at the end of June 1940, 62,000,000 cattle were under supervision for tuberculosis. At that time 230,000,000 tuberculin tests had been performed and 3,772,000 tuberculous animals had been slaughtered, the total cost being 86,000,000 dollars for administrative expenses and 188,000,000 dollars for compensation. The percentage of reactors showing no lesions on post-mortem was 0·22, and the report discusses the causes of the apparent non-specific reactions to tuberculin and emphasizes the need for controlling avian tuberculosis. It also urges the disinfection of all cattle trucks. In the year ending in June 1940, 12,220,000 tuberculin tests were performed and 56,343 cattle reacted (Wight (198)), this being the lowest percentage of reactors yet recorded. The number of herds fully accredited in 1940 was 271,700 containing 3,893,000 cattle. The effect of the control policy can be seen from the abattoir figures, where out of approximately 9,500,000 cattle killed only 8384 animals (in addition to those slaughtered as reactors) showed tuberculous lesions. Wight also draws attention to the prevalence of avian tuberculosis in poultry flocks, though his actual figures show that less than 5% of the flocks are affected. It is interesting to note (Duckworth (199)) that by November 1940 every state in the U.S.A. was a modified tuberculosis-free area, the last counties in California becoming accredited during that month. This means that throughout the U.S.A. the incidence of tuberculosis in cattle does not exceed 0·4%.

In Canada progress has been slower, but according to Hall (200) about half of the cattle in the country had been tested by the end of 1940 (3,919,991 out of 8,474,600) and a further 957,000 were awaiting test. From the figures then available Hall considers that not more than 2·0% of the cattle population is tubercular. In Sweden, Balteanu, Francke & Popa (201) suggest the creation of tuberculosis-free areas, starting in the Northern Provinces where all reactors would be slaughtered and none but free animals allowed entry. They also suggest the formation of protected areas in other districts in which the reactors would be marked but not slaughtered. Nielson (202) states that in Denmark the law that, if 90% of the herds in any district are tuberculosis-free, the remaining herds can be compulsorily tested and all reactors slaughtered, has been of no value owing to the opposition which it created. In 1937 the state offered compensation for the reactors and empowered dairies to reduce their payments to those farms which were not tested. The dairies undertook to test all herds in their areas for 10 years, and compensation was paid for all reactors slaughtered within 1 month of reacting. Between 1932 and 1939, ninety-five dairies entered the scheme, and between 1939 and 1941, 137. The scheme is thus making more progress than the previous compulsory law. In the Rhineland district of Germany a scheme was started in 1939 (Karmann (203)) under which all cattle and calves are tested by the intradermal test and the farms divided into three groups: class A farms are those which are tuberculosis-free and receive a bonus on the sales of milk; classes B and C farms are those with tuberculous animals, class C having a greater degree of infection than class B. Class C farms are paid a reduced price for their milk. During the 18 months the scheme had been operating the number of class A herds had increased from 546 to 673 and the numbers in the B and C classes had each fallen by nearly 100.

In Britain tuberculin testing under the Attested Scheme is done either by local practitioners or official veterinarians. The available figures at November 1942 show that a total of 16,222 herds were attested, 3910 in England, 8920 in Wales (mostly very small) and 3392 in Scotland. Steward (204) records the results of examining cattle for open tuberculosis under the Tuberculosis Order. He found the direct examination of a smear to be satisfactory, only one out of 400 cases recorded as positive by this method failing to show infection on post-mortem. If negative to the direct method Steward advises concentration of the expectorate and suggests that a 3% solution of Chloros (a hypochlorite) in 0·5% caustic soda should be used as the suspending fluid. As large a quantity of sputum as is possible is added to 5–15 ml. of this solution and shaken at intervals until dissolved. The liquid is then centrifuged for 10 min. at high speed and the supernatant poured off. A film is made of the deposit. 20% of the samples negative to the direct test proved positive to the concentration technique. The quantity of expectorate and the frequency of expectoration bear no relation to the degree of infection in the cow, and the expectorate has no typical appearances—it may be white, yellowish or golden. Steward examined seventy-nine samples of uterine or vaginal discharges, twenty-four being positive; sixty-four urine samples, nine of which were positive; and a number of superficial lymph nodes, in which there were very few bacilli. Examination of the faeces was of no practical value. Dixon (205) describes the method he uses to collect samples of sputum for examination. He scrapes dried sputum from the walls, adds any fresh sputum obtainable, and puts the whole into a test-tube containing lime water. The hard flakes of mucus swell and can be disintegrated by shaking: they are then strained off through muslin. The sample is centrifuged and a smear made from the deposit. Ritter (206) examined the bronchial secretions of 508 cows suspected of open tuberculosis: 219 were positive, and in twenty of these tubercle bacilli were also demonstrated in the milk. The blood serum of these 219 animals was also tested by the complement fixation test. It was found that all twenty of the cows with udder tuberculosis gave a positive reaction, but there were only sixty-eight positives out of the 199 other open pulmonary cases. The examination of bronchial secretions with removal of the 'open' cases, i.e. Ostertag's method, has been widely applied in Germany but is now being superseded by tuberculin testing. Nieberle (207) reports that Ostertag's method has failed and that the percentage infection in herds with tuberculosis is rising, since tuberculin tests have revealed that of 412,000 cattle 31% were affected. In Nieberle's view tuberculin testing and the building up of tuberculosis-free herds will have to be undertaken if any real progress is to be made.

Tuberculin

Seibert has continued his work on the purification of tuberculin. With Watson (208) and with Glenn (209) he has studied the electrical and filtration method of purification as distinct from the chemical. He isolated two types of polysaccharide by electrophoresis. One type did not migrate and the total N_2 present was only 0·2%. The other, which was present in much greater amounts, could not be freed of nitrogenous impurities so successfully, the minimum amount of N_2 being 0·85%. This substance migrated in the electric field. It is suggested that the protein is bound to the surface of the polysaccharide and that this causes the slight mobility. Seibert & Glenn (209) have prepared a standard P.P.D. tuberculin which is stored dry *in vacuo*, the preparation being modified by replacing the trichloracetic acid method of purification by an ultrafiltration method using collodion. The product so obtained is purer. It has a molecular weight of 10,500 and contains 92·9% protein, 1·2% nucleic acid and 5·9% polysaccharide.

The specificity of the extracts of acid-fast bacilli for their own types has been examined by Sahai (210), who sensitized groups of guinea-pigs by inoculating saline suspensions of the human, avian and vole types of *Myco. tuberculosis* and *Myco. johnei*, as well as suspensions in liquid paraffin of various saprophytic species of acid-fast bacilli. After sensitization, tests were made with decreasing concentrations of the various tuberculins or extracts at intervals between the 35th and 52nd day after inoculation. A high titre was obtained against homologous extracts and a smaller titre to heterologous, the liquid paraffin technique enabling a titre of 1:4000 and over to the homologous strain to be obtained by a dose of 2–3 mg. of the saprophytic bacteria. The vole strain extract did not give reactions in guinea-pigs sensitized against the saprophytic

types. Sahai suggests that this may be due to the extract being weak, since only one unstandardized extract was used. On the other hand, it may be noted that when tested on guinea-pigs inoculated with the homologous vole strain its titre was high. Such guinea-pigs also reacted strongly to mammalian tuberculin, whereas those inoculated with members of the saprophytic group, while exhibiting considerable cross reactions among themselves, reacted only slightly with mammalian tuberculin.

The causes of false positive tuberculin tests are being extensively studied and numerous reports have been published. In America and Britain avian tuberculosis and Johne's disease appear to be the usual causes, though certain other diseases have also been implicated. In Scandinavia the main difficulty still appears to be human tuberculosis.

The value of johnin and tuberculin for differentiating the two diseases is not certain. Johnson, Milligan & Cox (211) injected johnin into one caudal fold and tuberculin into the other. Of 347 cattle tested, twenty-four reacted to johnin and of these nine also reacted to tuberculin. None of the johnin-negative cattle were positive to tuberculin. The nine reactors were slaughtered and all were typical cases of Johne's disease, no evidence of tuberculosis being found. These authors also consider that mammalian tuberculin is not capable of distinguishing between the mammalian types of tuberculosis. Stenius (212) reports that the tuberculin commonly used in Finland is a mixture of the human and bovine types. When pure tuberculins were used he found that (*a*) in herds with bovine infection both human and bovine tuberculins gave equal reactions; (*b*) a similar result was recorded with herds free from bovine but exposed to human infection; and (*c*) in herds free from mammalian but exposed to avian infection, the avian type of tuberculin gave bigger reactions, which could be used for differentiation.

The present position regarding avian tuberculosis has been reviewed by Harbour (213), who states that this type of infection is very high in poultry in north Wales, about 20% of the adult birds received for post-mortem examination being infected. The risk of cattle infection in this district is therefore high. Feldman & Moses (214), on the other hand, tested 1595 cattle in forty-nine premises with mammalian and avian tuberculin. Fifty-one gave positive reactions. When slaughtered thirty-one were tuberculous, five had skin lesions and fifteen gave negative results to both guinea-pig and cultural examinations. In no case were avian-type tubercle bacilli recovered, although this infection was known to be present in fifteen of the forty-nine premises. Glover (215) examined post-mortem thirty-five cows, none of which were suspected of tuberculosis. Five gave definite evidence of avian tuberculosis, and in five there was strong evidence of this type of infection, though no bacilli could be recovered by cultural examination or animal inoculation.

Nielsen & Plum (216) have described the reactions of two herds to repeated tuberculin tests, including the use of P.P.D. The infection was finally traced to the human type, and in one herd the probable source of the infection was detected. In the other no source of infection could be found. The difficulty of tracing possible sources of human infection in cattle is also emphasized by Reid (217), who records that a heifer which had given contradictory results with the tuberculin test in different sites was found on post-mortem to be infected with the human type in the pharyngeal region. No source of infection could be traced. Nielsen & Plum (218) in a further communication suggest that human, bovine or 'intermediate' types of infection could all be transmitted to the bovine animal by contact with open respiratory tuberculosis in man. They give records of seventeen cases of bovine tuberculosis in cattle in tuberculin-tested herds which were infected from open pulmonary cases in the attendants. They stress the great difficulty of tracing such infection among suspected personnel. Another source of infection from the human has been recorded by Stenius (219), who traced infection in cattle to a brook contaminated with human tubercle bacilli from the waste effluent of a tuberculosis sanatorium.

Another source of positive tuberculin reactions and of tubercle-like lesions are certain *Actinomyces* and *Corynebacteria*. Lesions anatomically indistinguishable from 'skin lesions' of tuberculosis are described by Jöhnk (220) who found them in seventy-four cattle imported from Ireland. The lesions were mainly confined to the legs, especially to those parts where mechanical injury is likely to occur. The smaller lesions had a yellowish caseous centre, often calcified, and closely resembled the nodular lesions of tuberculosis, but the larger lesions resembled abscesses rather than tubercles, since they consisted of pus surrounded by a thick fibrous capsule. Though

the cattle reacted to the tuberculin test no evidence of tuberculosis was found post-mortem. Feldman & Moses (214) found that eight cattle which reacted to tuberculin were actually suffering from Actinomycosis or Actinobacillosis. The same authors (221) also report that in post-mortem examinations of 101 animals positive to the tuberculin test, bovine tuberculosis was demonstrated in only twenty-three animals; in twenty-two others with apparent tuberculous lesions no tubercle bacilli could be demonstrated, but in a further seventeen a ray fungus was demonstrated.

The reliability of tuberculin in detecting actual infected animals has been further investigated by several Australian workers. The tuberculin used was a synthetic medium tuberculin prepared locally. Legg & Maunder (222) carried out intradermal tests on 594 animals with this tuberculin and concluded (1) that two readings were required, but that if only one was practicable it should be at the 96th hour, and (2) that the test with this tuberculin was efficient in cases where the disease was well established but less efficient where there were only small lesions or where the disease was very advanced. A further 271 animals were tested simultaneously with this tuberculin, with a Canadian synthetic tuberculin and with a concentrated old tuberculin. A single inoculation was used for the two former and a double one for the latter. Of the thirty-two tuberculous animals twenty-seven were positive to the Canadian, twenty-seven to the old tuberculin and twenty-eight to the local product. All the animals were examined post-mortem. The same authors (223) reported on the field use of a tuberculin prepared by the same laboratory but concentrated by ultrafiltration. All 458 cows tested were examined post-mortem. Positive tuberculin reactions had been recorded in three out of 416 in which no lesions could be demonstrated, in fourteen out of sixteen with small lesions, in all of the sixteen with moderate lesions and in five out of ten with generalized T.B. Bazeley & Barraclough (224) performed similar tests on 1595 cattle using three local batches of tuberculin, the U.S.A. Bureau of Animal Industry tuberculin and a Canadian product. All were equal in regard to potency, clarity and ease of reading and efficiency, except for one local batch which did not compare favourably with the rest.

Bull (225) discusses these recent Australian results and suggests that, in view of the negative reactions given by many of the advanced cases, it might be of advantage if a reliable complement-fixation test could be evolved to supplement tuberculin testing.

An official review of the position with regard to tuberculin testing in Great Britain has been issued by the Ministry of Agriculture (226). They state that the present tuberculin is based on that issued by the Bureau of Animal Industry in the U.S.A. but that it will shortly be replaced by P.P.D. One practical point is strongly emphasized, i.e. that the skin of the neck varies greatly in its reaction to tuberculin, the portion nearer the head reacting more strongly than that nearer the shoulder. The method to be adopted when performing a comparative test is given in detail.

A possible danger in tuberculin testing in herds containing a number of tuberculous cattle is discussed by Marcusson (227), who submits evidence that the injection of tuberculin causes a flare up of latent injections and that open clinical infection often follows tuberculin injections. He states that this is known to occur in the human, and that the dose of tuberculin used in cattle is nearly 100 times that used in human practice. He considers that all reactors should be removed from the herd as soon as possible; otherwise the increase in open cases will lead to increased herd infection.

Vaccination

Benzoni (228) inoculated 100 white mice with B.C.G. and an anatuberculin vaccine. Both conferred some resistance, but B.C.G. was the more effective. The immunity was more definite, was produced more rapidly and was more permanent. B.C.G. vaccination in calves appears to be of value if the infecting dose is not administered too soon after vaccination and is not repeated too frequently. Buxton, Glover, Dalling & Bosworth (229) vaccinated eight calves with 100 mg. of B.C.G. followed by 50–100 mg. every 6 months. After 3 years they were infected *per os* with 5 mg. of virulent bovine tubercle bacilli and were confined in close contact with animals known to be tuberculous. After 6 months they were killed and five were found to be completely free of lesions, while the other three had only slight signs of tuberculosis. In a second

group of twenty calves which were vaccinated each 6 months for 3 years, each vaccination, being followed 2 months later by an infecting dose, no evidence of resistance was found in the vaccinated calves on post-mortem. Similarly, a third group of ten calves which were first vaccinated three times and then repeatedly vaccinated and infected showed no resistance. The evidence on B.C.G. available during the last 15 years has been summarized by Paraf (230), who states (1) that it is harmless, such disasters as the Lubeck outbreak being due to accident; (2) it is stable and cannot be changed experimentally to a virulent type; (3) it is efficient, for cattle, monkeys and rabbits have been protected against inoculation of virulent cultures, while even the very susceptible guinea-pig has been rendered semi-resistant; and (4) while the oral route is possible, parenteral injection is better and scarification best.

The scarification method is also advocated by Birkhaug (231), who states that the reaction is slight and that there is no suppuration of the local glands or visible scars. Experimentally the tuberculin reaction becomes positive in 2–4 weeks, by which time resistance to infection with a virulent organism is definite. The positive tuberculin reaction remains for at least 14 months. This method or the multiple skin prick methods of Rosenthal (232) and Weil Halle (233) have been further studied by Nègre & Bretey (234), who used an emulsion of B.C.G. (5 mg./ml.) for the vaccine. A drop of this emulsion was placed on the shaved skin of the backs of seventeen guinea-pigs and each was pricked 30 times. After 8 days eight reacted to tuberculin and after 1 month seven out of eleven survivors reacted. These seven, with ten controls, were tested for immunity by the subcutaneous injection of 0·0005 mg. of virulent *Myco. tuberculosis*. Only three of the seven survived for 6 weeks, when they and the controls were killed. Lesions were advanced in the controls and almost absent in the three vaccinated animals. It was found that slightly better results were obtained when sixty pricks were made. Scarification using six scarifications each 1 cm. long was used to produce resistance in fifteen guinea-pigs. Allergy was produced in six animals after 8 days and in seven out of nine survivors after 1 month. These survivors, with eight controls, were tested by injection of *Myco. tuberculosis* and killed after 6 weeks. The controls were grossly diseased, but the vaccinated animals showed no lesions in the lungs or liver and the spleens were either normal or only slightly affected. Another eleven guinea-pigs were vaccinated by eight repeated scarifications at 3-day intervals. Seven of the eleven showed an allergic response 8 days after the beginning of the vaccination and all ten survivors at the end. Eight survivors and eight controls were tested with virulent bacilli and the six vaccinated guinea-pigs which survived for 7 weeks were free from gross lesions. All the controls were heavily infected. These results are claimed to confirm the value of the skin prick method of vaccination with B.C.G. and to show the value of this vaccine. It is noticeable, however, that although the organs of the vaccinated animals showed few lesions, the death rate from intercurrent disease in each group of vaccinated animals was high, whereas there were no deaths in their respective control groups. Vaccination appears therefore to lower the resistance of the animals to other diseases even if it raises it to tuberculosis.

According to Liebow, Burn & Soper (235) the incidence of intercurrent infection in guinea-pigs during and immediately following vaccination is higher in animals vaccinated with heat-killed bacilli than it is when B.C.G. is used as the vaccine. They also found that vaccination with B.C.G. appeared to confer some degree of resistance on the guinea-pigs, as judged by the delay in appearance and lesser extent of the lesions and also by the longer period of survival. They found that heat-killed bacilli of the S form gave some slight protection but those of the R form gave none. Whatever slight action heat-killed organisms may have in producing resistance to tuberculosis is said by Damerow (236) to depend on the *p*H of the suspending fluid, an alkaline reaction being necessary.

B.C.G. when inoculated intracutaneously in very small amounts into man (0·1 mg.) produced an allergy which, while less marked than that in naturally infected tuberculosis subjects, is nevertheless very definite and can be demonstrated a year after inoculation. Aronson, Parr & Saylor (237) found that of 1383 tuberculin-negative humans injected with this dose of B.C.G. 93·3% gave a positive tuberculin reaction a year later; of 1312 similar subjects not inoculated only 12·7% reacted. Griffith, Dalling & Pagel (238) suggest that the immunizing power of the vole-type bacillus is greater than B.C.G. In three calves the intravenous injection of from 0·01 to 10·0 mg. produced only slight tuberculous lesions which tended to disappear without

trace; a dose of 80 mg. intramuscularly produced a large local lesion but no generalization. This strain was then used to vaccinate a number of calves. Of these four were vaccinated once and five twice, their resistance to infection together with that of two controls being tested by the oral administration of 7·5 mg. of virulent culture. When killed about 200 days later none of the vaccinated animals showed more than slight lesions, though even in those with no visible tuberculous foci tubercle bacilli could be cultured from the apparently normal glands. The controls both showed widespread tuberculosis. This confirms the work of Wells & Brooke (239) on guinea-pigs. These authors found that small doses of the vole bacillus produced small retrogressive lesions and that when 2 mg. was used to vaccinate the animals these, when killed 11 weeks after infection with minimal doses of virulent bacilli, showed either no or slight lesions. On the other hand, animals vaccinated with B.C.G., even in larger doses, showed generalized tuberculosis though this was less severe than in the unvaccinated controls.

P. S. WATTS

THE HANNAH DAIRY RESEARCH INSTITUTE
KIRKHILL, AYR, SCOTLAND

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